The Genetics of Adaptation to a Harsh Granite Outcrop Environment in *Mimulus*

by

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Lisa Donovan

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Closely related populations or species often occupy ecologically disparate habitats. Adaptation to new habitats can maintain genetic variation within a species or eventually lead to speciation. Local adaptation to different environments has been repeatedly demonstrated in plants and animals, however the traits and genes that underlie this adaptation are poorly understood. This is because many traits differ between divergent populations and species. One way to solve this problem is to separate a trait from its genetic background through genetic manipulation and look for differences in fitness between genetically manipulated individuals.

My dissertation focuses on investigating the traits and genes that allow two species of Monkey flower, *Mimulus laciniatus* and *Mimulus filicifolius*, to survive in a unique habitat. Most closely related *Mimulus* species, such as *M. guttatus,* occur in streams and seeps, but *M. laciniatus* and *M. filicifolius* have each colonized a harsh granite outcrop environment. Another unique characteristic that both these species share is a lobed leaf shape. Because of the physiological properties of lobed leaves they should be adaptive in a dry, exposed granite outcrop. *M. laciniatus* also flowers earlier than nearby *M. guttatus* and is a small flowered self-fertilizing species while *M. guttatus* has large flowers and is highly outcrossing. Early flowering allows plants to escape the

onset of seasonal drought while a self-fertilizing mating system and small flower size is often correlated with the occupation of harsh habitats.

In chapter one I describe a new granite outcrop endemic species of *Mimulus, M. filicifolius* based on morphological divergence from *M. laciniatus*. *M. filicifolius* was previously categorized as *M. laciniatus* but it is geographically disjunct and its leaves are more finely dissected (Sexton, Ferris, and Schoenig 2013). In the second chapter I explore whether *M. filicifolius* is genetically divergent and reproductively isolated from *M. laciniatus* using genetic sequence, microsattelite, and hybrid fertility data from four members of the *M. guttatus* species complex with highly overlapping geographic ranges: *M. guttatus, M. nasutus, M. lacinaitus,* and *M. filicifolius*. In the third chapter I investigate the genetic basis of leaf shape differences in three members of the *M. guttatus* species complex, *M. laciniatus, M. nudatus,* and *M. guttatus* using bulk segregant analysis to map quantitative trait loci. In the fourth and final chapter I examine the genetic basis of flowering time, floral size, and leaf shape divergence between sympatric *M. guttatus* and *M. laciniatus* populations in a common garden using quantitative trait locus (QTL) mapping, phenotypic selection on flowering time, flower size, and leaf shape in *M. laciniatus x M. guttatus* hybrids in a reciprocal transplant experiment in the field, and whether QTL's from my common garden experiment overlap fitness QTL's in the field by genotyping hybrid individuals that survived to flower in the field.

Dedication

I would like to dedicate this dissertation to my family who has always loved and supported me. In particular I would like to dedicate this to my husband and best friend Alex. He has been through the rollercoaster with me and has never let go of my hand.

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1. THE FERN-LEAVED MONKEYFLOWER (PHRYMACEAE), A NEW SPECIES FROM THE NORTHERN SIERRA NEVADA OF CALIFORNIA

1.1 Summary

The fern-leaved monkey flower, ***Mimulus filicifolius*** (Phrymaceae, Section Simiolus), is a new species described from the northwestern corner of the Sierra Nevada of California. The new taxon is differentiated from close relatives of *Mimulus* L. (*M. laciniatus* Gray and *M. guttatus* DC.) mostly by having many finely divided, bi-pinnately compound leaves. ***Mimulus filicifolius*** occurs mainly within ephemeral seeps of rock outcrops, where it occupies similar habitats to *M. laciniatus*, which occurs farther south in the Sierra Nevada. ***Mimulus filicifolius*** appears to be highly geographically restricted, and is currently known only from Butte and Plumas Counties within the Plumas National Forest. It therefore merits strong conservation consideration.

1.2 Introduction

The genus Mimulus L. (Phrymaceae) is a diverse plant group that has its center of diversity in western North America (Grant 1924). Within this group, section Simiolus contains a variety of species that inhabit a wide array of habitats, from coastal areas to high mountains, and has become a focal group of interest in ecological and evolutionary studies (Wu et al. 2007). Within section Simiolus, the *Mimulus guttatus* DC. species complex comprises a group of morphologically differentiated, yet often interfertile

species (Vickery 1964). Here we describe a new species that is distinguished within section Simiolus mainly by having finely divided leaves, specimens of which were previously determined as *Mimulus laciniatus* A. Gray.

Species having divided leaves and leaf margins are rare within the genus *Mimulus*. *Mimulus guttatus* can have toothed margins, especially near the leaf base (Grant 1924), yet leaves of *M. guttatus* are mostly entire. Simiolus specimens having very finely divided leaves have been collected in and near Plumas National Forest since 1974 (W. Dakan CAS871913). *Mimulus laciniatus* was previously the only known member of *Mimulus* to have strongly dissected leaf margins (Grant 1924). *Mimulus laciniatus* is an annual plant endemic to the central western slope of the California Sierra Nevada where it primarily occupies ephemeral granite seeps at elevations generally > 900 m (Sexton et al. 2011). *Mimulus laciniatus* leaf divisions extend throughout the leaf, forming a laciniate or pinnately compound shape. The *M. laciniatus* species range is found between Tulare and Amador counties from south to north, respectively, but the morphologically distinct taxon described here (previously described as *M. laciniatus*) occurs approximately 150 kilometers north of the nearest known populations of *M. laciniatus* (Fig. 1).

Butte and Plumas County specimens, previously determined as *M. laciniatus*, differ morphologically from *M. laciniatus* mainly by having leaves that are finely twice-pinnately compound and having more primary leaf divisions, giving the leaves a

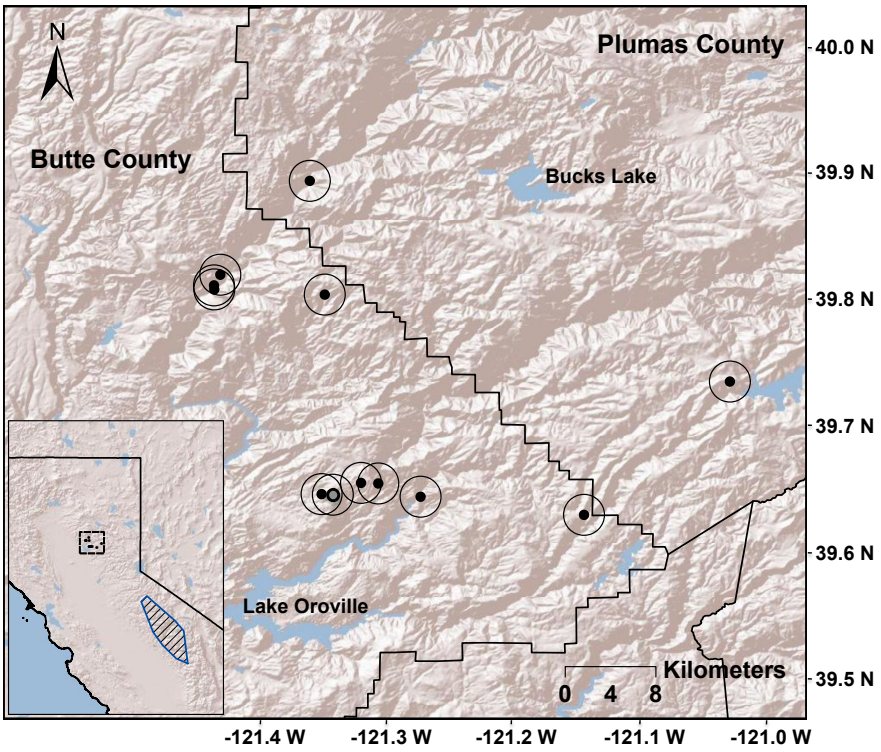

Figure 1. Species distribution of known *Mimulus filicifolius* locales (crosses) within Butte and Plumas Counties (dashed-line box within map inset), of the northwestern Sierra Nevada of California. The star represents the location of the *M. filicifolius* type specimen at Big Bald Rock (39° 38' 39" N, 121° 20' 36" W). The species range of the morphologically similar *Mimulus laciniatus* in the central Sierra Nevada is shown as the polygon with diagonal lines in the map inset.

delicate, fern-like appearance. Molecular genetic analyses indicate that the Butte Co. subpopulation from which the type specimen described here originates is genetically distinct from the *M. laciniatus* clade, and reproductive barriers in the form of hybrid sterility exist between this subpopulation and *M. laciniatus* and *M. guttatus* populations (Ferris et al., unpublished data). First-generation hybrids between individuals from this

new taxon and *M. guttatus* and *M. laciniatus* individuals exhibited hybrid sterility, whereas there is no comparable barrier between the same *M. laciniatus* and *M. guttatus* individuals. The above evidence of strongly differing morphological characters, reproductive barriers, and evidence of divergent evolution leads us to conclude that the northern Sierran plants previously identified as *M. laciniatus* should be treated as a distinct species.

1.3 Taxonomic Treatment

Mimulus filicifolius J. P. Sexton, K. G. Ferris and S. E. Schoenig, sp. nov. TYPE: U.S.A. California: Butte County, granite seeps of easterly area of Big Bald Rock, 39° 38' 39" N, 121° 20' 36" W, ca. 930 m elevation, 22 May 2010, J. P. Sexton 1 (holotype, DAV). Fig. 2.

Mimulus filicifolius is distinguished from *M. laciniatus* A. Gray by having strongly bi-pinnately and finely divided—often linear—leaf margins in larger plants, and more primary leaf divisions (often having 8 or more primary leaf divisions on one leaf side) (Fig. 2C & 3), as opposed to having mostly laciniate to occasionally bi-pinnate leaf shapes (with 7 or less primary leaf divisions on one leaf side, often 3 or less) with oblanceolate lobes; having clasping, entire, ovate floral node bracts, as opposed to having bract bases long-tapered to petioled, and bracts narrowly lanceolate to pinnately lobed; and having pedicels less than 2 times the calyx length, as opposed

Table 1. ***Diagnostic morphological characters between Mimulus filicifolius and M. laciniatus.***

Trait	*M. filicifolius*	*M. laciniatus*
Leaf shape	Pinnate to strongly bi-pinnate, having fine, linear lobes; often having 8 or more primary pinnae on a side	Laciniate to bi-pinnate, lobes oblanceolate, ≤ 7 primary pinnae on a side and often having 3 or less.
Floral bracts	Clasping, ovate, entire	Base long-tapered to petioled, lanceolate to pinnately lobed
Pedicels	Relatively short, < 2 times calyx length	Relatively long, often ≥ 2 times calyx length

to often having pedicels equal to 2 times the calyx length or longer (Table 1, Fig. 3).

Herbaceous annual, 3-38 cm, glabrous throughout. **Leaf petioles** 0-32 mm**, leaf blade** 3-68 mm, oblanceolate to ± ovate, bi-pinnately, narrowly to finely lobed (linear) or dissected, often having > 8 primary pinnae divisions on a side. **Inflorescence** a raceme, generally > 5-fld; bracts clasping at base, entire, ovate. **Flowers** open, occasionally cleistogamous; **pedicel** 2.5-14 mm; **calyx** 2-11 mm, strongly curved (rounded), asymmetrically swollen in fruiting, ± glabrous, lobes unequal, lowest 2 upcurved in fruiting; **corolla** pale yellow, tube-throat 4-8 mm; **placentas** axile. **Fruit** 3-8 mm, ovoid to

Table 2. Leaf shape and pedicel/calyx length ratio data were recorded from herbarium sheets for the nine known locales of Mimulus filicifolius and 12 locales of Mimulus laciniatus. Herbarium code, specimen number and the number of individual plants examined from each herbarium sheet given. CAS = California Academy of Sciences; CHSC = Chico State Herbarium, California State University, Chico; DAV = University of California, Davis Center for Plant Diversity; JEPS = Jepson Herbarium.

Specimen ID	Species	Locale	*N* (leaf)	*N* (pedicel)	Lat.	Long.
CHSC39058	*M. filicifolius*	Bald Rock Dome, Butte Co., CA, USA	6	9	39.6536	-121.307
CHSC40889, DAV189651	*M. filicifolius*	Bean Creek Road, near Little Bald Rock, Butte Co., CA, USA	4	4	39.6539	-121.32
CAS871914, CHSC33342	*M. filicifolius*	Big Bald Rock, Butte Co., CA, USA	2	2	39.645	-121.352
DAV190412, DAV190658, DAV190659	*M. filicifolius*	Big Bald Rock, Butte Co., CA, USA	4	5	39.6445	-121.343
CHSC50115	*M. filicifolius*	Feather Falls Trail, Butte Co., CA, USA	4	3	39.6431	-121.273
CAS916469, CHSC42866	*M. filicifolius*	Lumpkin Ridge, Butte Co., CA, USA	9	8	39.6286	-121.144
CHSC94564	*M. filicifolius*	Poe Dam area, Feather River, Butte Co., CA, USA	1	1	39.8072	-121.437
CHSC49002	*M. filicifolius*	Western Pacific Railroad between Pulga and Poe Dam, Butte Co., CA, USA	2	2	39.8106	-121.437
CAS871913	*M. filicifolius*	North Fork Feather River, Plumas Co., CA, USA	2	2	39.8933	-121.361
JEPS10456	*M. laciniatus*	Yosemite National Park, Mariposa Co., CA, USA	-	4	-	-
JEPS10937	*M. laciniatus*	Hog Ranch, Tuolumne Co., CA, USA	6	7	37.8822	-119.855
JEPS10938	*M. laciniatus*	Dardanelle, Tuolumne Co., CA, USA	5	7	38.3411	-119.833
JEPS11022	*M. laciniatus*	Yosemite Falls, Mariposa Co., CA, USA	1	7	-	-
JEPS11025	*M. laciniatus*	Strawberry Lake, Tuolumne Co., CA, USA	4	6	38.1954	-119.981
JEPS11026	*M. laciniatus*	Marble Fork, Sequoia NP, Tulare Co., CA, USA	1	2	36.5534	-118.81
JEPS23793	*M. laciniatus*	Jose Basin, Fresno Co., CA, USA	9	9	37.1014	-119.374
JEPS33899	*M. laciniatus*	Mono Hot Springs Campground, Fresno Co., CA, USA	8	9	37.3267	-119.017
JEPS53950	*M. laciniatus*	Vermillion Valley, Fresno Co., CA, USA	9	7	37.4081	-118.938
JEPS55430	*M. laciniatus*	Miramonte, Fresno Co., CA, USA	3	3	36.6925	-119.051
JEPS6975	*M. laciniatus*	Mills Creek, Fresno Co., CA, USA	7	8	37.4244	-118.858
JEPS82859	*M. laciniatus*	Clover Creek, Tulare Co., CA, USA	4	4	36.6019	-118.743

fusiform, loculicidal (indehiscent), chambers 1-2; **seeds** many, generally < 1 mm, ovoid, ± yellow to dark brown.

1.4 Paratypes Examined

We examined all of the known herbarium specimens of *M. filicifolius*, including paratypes (Table 2). The following paratypes (herbarium and specimen codes are given in parentheses) are from the *M. filicifolius* geographic range and were previously identified as *M. laciniatus*: U.S.A. California, **Butte County:** South of Lumpkin Ridge, 12 May 1987, L. Ahart 5634 (CAS916469, CHSC42866); Fall River at the head of Feather Falls, 30 April 1990, V. Oswald 4175 (CHSC50115); Along Bean Creek Rd. near Little Bald Rock, 22 May 1985, L. Ahart 5027 (CHSC 40889), 7 June 2009, D. Grossenbacher and M. James 1032-a (DAV189651); Big Bald Rock, 14 June 1980, R. Banchero 220 (CAS871914, CHSC33342); Bald Rock Dome, 15 May 1983, R. Schlising 4414 (CHSC39058); Between Pulga and Poe Dam near the North Fork of the Feather River, 11 September 2006, L. Ahart 13293 (CHSC49002); Poe Dam area, 26 April 1986, V. Oswald 1981 (CHSC94564). **Plumas County:** North Fork Feather River ½ mile below the mouth of Rock Creek, between Storrie and Elephant Butte Tunnels, 28 April 1974, W. Dakan (CAS871913).

Figure 2. A) *Mimulus filicifolius* prior to flowering, growing near Feather Falls, Butte Co., California, 12 May 2012. Photo by S. Schoenig. B) *Mimulus filicifolius* flowering at basalt site south of Lumpkin Ridge, Butte Co., California. Photo by Robert Schlising. C) *Mimulus filicifolius* leaves from plants growing at Big Bald Rock, Butte Co., California. Photograph by S. Schoenig. Scale bar = 5 mm.

1.5 Morphological Analysis

We quantified differences in leaf shape and pedicel length between *M. filicifolius* and *M. laciniatus* from herbarium specimens. Collections included all known locales (9) of *M. filicifolius* from Butte and Plumas counties, and 12 locales from 4 counties

representing much of the species range of *M. laciniatus* (Table 2). We recorded data from each plant having clearly observable traits on a herbarium collection sheet (Table 2). Only complete individuals (i.e., having attached roots or being the only specimen on a sheet) were counted. For leaf shape, we recorded the greatest number of primary divisions on one side of the longest leaf on a plant. Leaf margin lobes near the leaf tip were included in counts since it was difficult to distinguish primary and secondary pinnae there. A total of 34 and 57 individuals were measured for leaf shape in *M. filicifolius* and *M. laciniatus*, respectively. For pedicel length, we measured the longest pedicel and its associated calyx on a given plant and recorded the pedicel/calyx length ratio. We measured a total of 36 and 73 individuals for pedicel/calyx length ratios for *M. filicifolius* and *M. laciniatus*, respectively.

Morphological data were analyzed using REML (JMP, version Pro 10). The effect of species was considered a fixed factor, whereas population (locale) was considered a random factor nested within species since we were primarily interested in species differences. Species differences were highly significant for both leaf and pedicel traits. For leaf shape, *M. filicifolius* and *M. laciniatus* had least square means of 8.23 (± 0.78 SE) and 2.52 (± 0.65 SE) primary pinnae, respectively (df = 1; error df = 17.29; $F = 31.75$; $P < 0.0001$; Fig. 3a). For pedicel length, *M. filicifolius* and *M. laciniatus* had least square means

of 1.15 (± 0.32 SE) and 3.23 (± 0.24 SE) pedicel/calyx length ratios, respectively (df = 1; error df = 16.89; $F = 26.91$; $P < 0.0001$; Fig, 3).

1.6 Distribution and Habitat

The epithet ('fern-leaved' in Latin) for the new species refers to its strong and finely compound leaf structure (Figs. 2C). Mimulus filicifolius is known between 430-1280 m within the Feather River watershed of the northern California Sierra Nevada (Fig. 1) and most specimens are known from slow-draining, ephemeral seeps of the Bald Rock Pluton in Butte County (e.g., Big Bald Rock, Little Bald Rock, and Bald Rock Dome), with noted exceptions (e.g., a locality on Lovejoy basalt at Lumpkin Ridge). These habitats are mainly comprised of exfoliating granite slabs on which mosses and club mosses grow and occur within a mixture of chaparral and yellow pine forest, dominated by Arctostaphylos viscida Parry, Quercus chrysolepis Liebm., Quercus kelloggii Newb., Pinus ponderosa ex Lawson and C. Lawson, and Pseudotsuga menziesii (Mirb.) Franco. Noted native plant associates of *M. filicifolius* at Big Bald Rock include species of *Bryum* Sendtn. ex C. Müll., *Cheilanthes gracillima* D. C. Eaton, *Heterocodon rariflorum* Nutt., *Penstemon newberryi* A. Gray, and *Selaginella wallacei* Hieron. Flowering specimens of *Mimulus filicifolius* have mostly been collected or observed from April to June, with one specimen collected in September (L. Ahart 13293, CHSC94564).

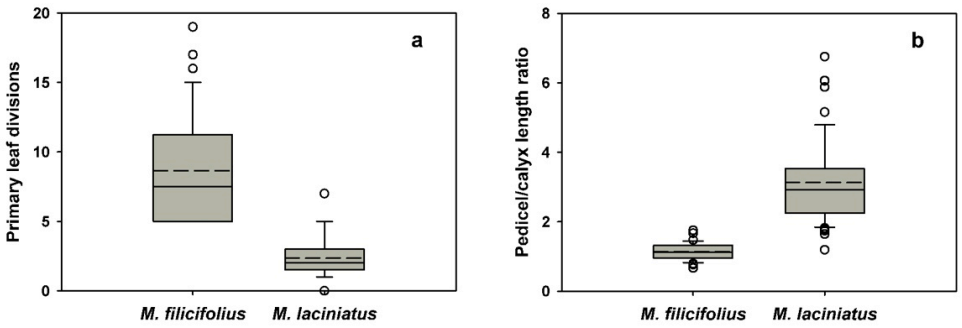

Figure 3. Box plots of morphological data of distinguishing characters between *Mimulus filicifolius* and *Mimulus laciniatus*. (a) Number of primary leaf divisions (range = 5-19 and 0-7 for *M. filicifolius* and *M. laciniatus*, respectively). (b) Pedicel/calyx length ratio (range = 0.667-1.75 and 1.19-6.75 for *M. filicifolius* and *M. laciniatus*, respectively). Box boundaries are 25th and 75th percentiles. Dashed centerline is the mean; unbroken centerline is the median. Whiskers are 90th and10th percentiles. Unfilled circles are points outlying.

1.7 Conservation Considerations

Mimulus filicifolius is endemic to the northwestern corner of the California Sierra Nevada and is known from only nine locales on or adjacent to the Plumas National Forest, several of which are closely spaced (Fig. 1). We did not perform extensive searches to locate new populations within suitable habitat. Besides Big Bald Rock, the type specimen locale, we visited several other locales from 2006 to 2012 to observe habitats and the range of phenotypes from several sites across the species range. The locale visited at Big Bald Rock appeared to be healthy (containing thousands of individuals). Nevertheless, we were unable to locate plants at the paratype locales near Pulga and Poe Dam near the North Fork of the Feather River from which specimens had

been collected by L. Ahart 13293 (CHSC49002) and V. Oswald 1981 (CHSC94564). L. Ahart described the population at this locality as "uncommon, only one plant seen." Additionally, at the paratype collection site near Little Bald Rock, *M. filicifolius* was described as "uncommon," although we did not visit this locale. The population that we observed at Feather Falls Trail was fairly small, consisting of perhaps a few dozen individuals adjacent to a scenic overlook. Since there are few known populations, some of which are small and occur close to each other, we recommend that conservation managers include this species in monitoring programs to limit future risks to existing populations (e.g. species invasions, land clearing, livestock introductions). Additionally, suitable habitats within the region should be searched in case other populations exist.

1.8 Discussion

We find no evidence that *M. laciniatus* occurs within the species range of *M. filicifolius*. All specimens known from Butte and Plumas counties are consistent with the *M. filicifolius* phenotype and it appears from our analysis that these two taxa are strongly diverged geographically and evolutionarily.

Mimulus filicifolius has a lobed leaf shape similar to, but more finely dissected than, *M. laciniatus. Mimulus filicifolius* and *M. laciniatus* also occupy similar habitats—seeps in rocky outcrops. *M. laciniatus* has been shown to be adapted to these habitats compared to its close relative, *M. guttatus* (Peterson et al., in press). Since *M. filicifolius* is

genetically distinct from *M. laciniatus* (Ferris et al., unpublished data) its leaf shape may be an independent derivation of lobed leaves in the genus *Mimulus*, which would represent parallel phenotypic evolution in parallel environmental conditions and thus be strong evidence of adaptation.

A lobed leaf shape may be adaptive in exposed, outcrop environments because it may help reduce heat stress and water loss in the daytime and/or reduce cold stress at night. Rock outcrops are drier, more light-intensive and have more extreme ground temperatures than the longer-lasting seep and stream habitats of nearby Simiolus species such as *M. guttatus* or *M. nasutus* (K. Ferris unpublished data). Lobed leaves have thinner boundary layers than round leaves, which increases the efficiency of convective heat transfer. Heat loss through convection can reduce the amount of water lost to evaporative cooling in hot, dry environments like rocky outcrops (Givnish 1978; Schuepp 1993; Nobel 2005; Nicotra et al. 2011).

Lobed leaves may also contribute to freeze tolerance early in the growing season when nights are still cold. On clear nights, leaves in exposed, open areas like *M. filicifolius* and *M. laciniatus* habitats radiate heat to the cold sky. This radiation can cause leaf temperatures to fall below air temperature by several degrees and thus leaves can freeze when air temperatures are near, but still above 0° C (Darwin and Darwin 1880; Nobel 2005). Because of their reduced boundary layer lobed leaves should stay closer to

air temperature than round leaves, and thus warmer at night. Because of the above physiological effects lobed leaves in *M. filicifolius* and *M. laciniatus* could be a key adaptive trait in the rocky outcrop environments they occupy, although we acknowledge that these hypotheses remain to be rigorously tested.

2. SPECIATION OF GRANITE OUTCROP ENDEMICS IN THE *MIMULUS GUTTATUS* SPECIES COMPLEX

2.1 Summary

Speciation can occur on both large and small geographic scales. In plants it is thought that local speciation, where small populations split off from a large ranged progenitor species, is the dominant mode. Plants may be particularly adept at speciation in small populations because of their ability to adapt to local environmental conditions and the frequent evolution of self-fertilizing mating systems. A recently described morphological species in the genus *Mimulus*, *Mimulus filicifolius,* is an excellent candidate for local speciation because of its highly restricted geographic range. *M. filicifolius* was formerly described as a geographically disjunct population of *M. laciniatus,* a member of the *Mimulus guttatus* species complex, because of its similar lobed leaf morphology and granite outcrop habitat. In this study we investigated whether *M. filicifolius* is genetically distinct and reproductively isolated as well as morphologically distinct from *M. laciniatus.* We examined patterns of genetic variation in 7 nuclear loci, determined genotypes at 8 microsatellite markers, and measured hybrid fertility in *M. filicifolius* and *M. laciniatus,* along with two other members geographically proximate members of the *M. guttatus* species complex*: M. guttatus* and *M. nasutus.* We found that *M. filicifolius* is genetically distinct from all three other species and that it is both pre and post-zygotically reproductively isolated from *M. laciniatus*. We conclude that *M.*

filicifolius is a truly distinct new species with a small geographic range that is consistent with it being a product of local speciation.

2.2 Introduction

Most species are formed in allopatry, but allopatric speciation can occur on a variety of geographic scales. Speciation may happen on a broad scale such as when a species is split by a cataclysmic geographic event like the uplift of a mountain range or the rise of the Isthmus of Panama. The populations on either side of the divide will diverge, eventually forming new sister species (Jordan 1908, Mayr 1954b, Knowlton and Weight 1998, Lessios 1998). Alternatively speciation could occur on a local geographic scale with small populations splitting off and diverging from a large progenitor species' range (Mayr 1963, Grant 1971, Carson and Templeton 1984, Barraclough and Vogler 2000, Gottlieb 2003). In plants it has been argued that speciation happens mostly in small populations (Stebbins 1950, Clausen 1951, Levin 1993, Rieseberg and Brouillet 1994, Gottlieb 2003). One reason given for this is that plants' sessile lifestyle restricts gene flow geographically compared to animals (Raven 1980, Raven 1986, Levin 1993). Frequent adaptation to local conditions (reviewed in Hereford 2009, Kawecki and Ebert 2004) and the repeated evolution of self-fertilizing mating systems (Stebbins 1950) also seem highly relevant to the pervasiveness of local and budding speciation in plants. If plant populations often adapt to local conditions, then it is logical to suppose that differential

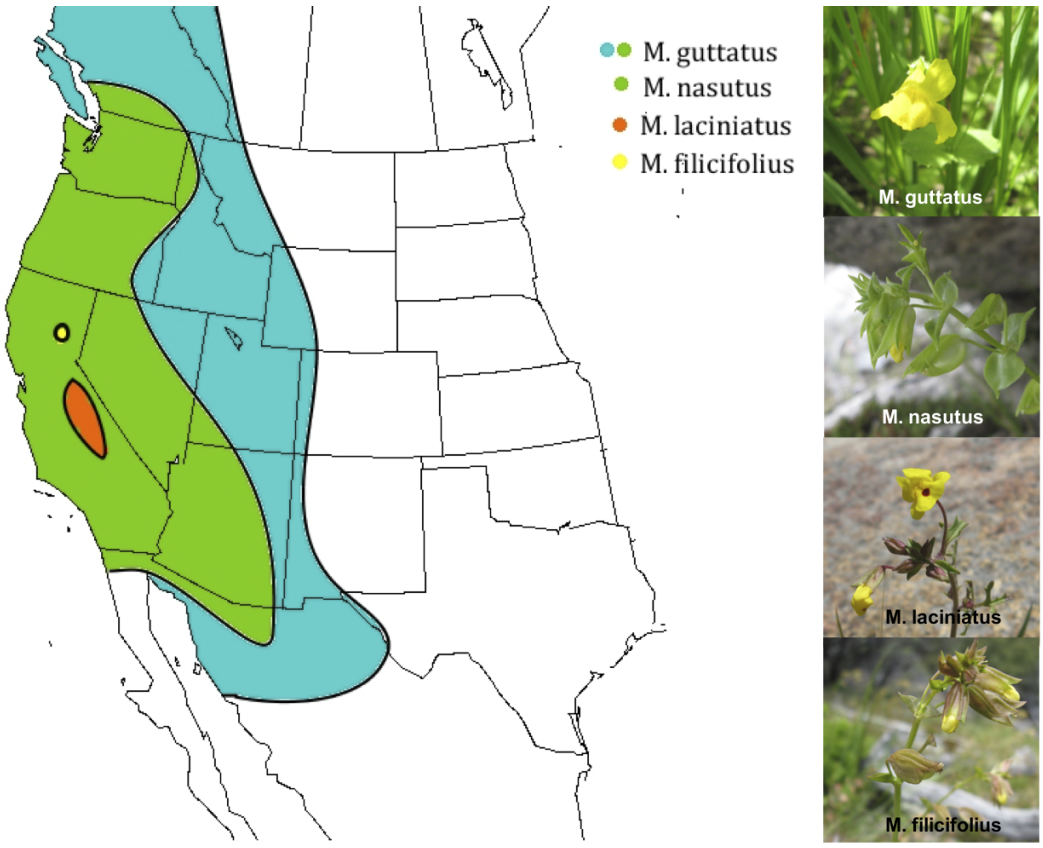

Figure 4. Geographic distributions of *M. guttatus (blue and green)*, *M. nasutus (green)*, *M. laciniatus (orange)*, and *M. filicifolius (yellow)*. *M. guttatus's* distribution encompasses the ranges of the other three taxa.

adaptation might lead to forms of reproductive isolation, and eventually speciation. Self-fertilization assures the reproductive success of colonizing plants in the absence of con-specific neighbors (Baker 1955) and maintains favorable combinations of locally adaptive alleles (Antonovics 1968, Jarne and Charlesworth 1993). Self-fertilization is also an effective reproductive isolating barrier from a nearby outcrossing relative (Kiang and Hamrick 1978, Fishman and Wyatt 1999, Martin and Willis 2007, Levin 2010).

One of the best-studied examples of local speciation in plants is that of the species pair *Stephanomaria exigua ssp.coronaria* and *Stephanomaria malheurensis* (Gottlieb 1973b, 1976, 1979, and 2003). *S. exigua ssp. coronia* is a highly outcrossing species with a large geographic range extending from southern California through Oregon while *S. malheurensis* only occurs at one location at the very northern end of *S. exigua's* range (Gottlieb 2003). The two species are highly similar morphologically, but using allozymes Gottlieb determined that the self-fertilizing *S. malheurensis* contains a distinct subset of the genetic variation present in the outcrossing *ssp. coronaria* indicating that *S. malheurensis* is a recent derivative of *ssp.coronia*. He also found that the two species were reproductively isolated by differences in mating system, chromosomal rearrangements, and Dobzhansky-Muller incompatibilities despite genetic and geographic proximity (Gottlieb 1973b). In his 2003 review of local, or as he calls it progenitor-derivative, speciation Gottlieb argues that this type of speciation is common in plants.

The *Mimulus guttatus* species complex is a closely related group of morphologically and ecologically diverse species with numerous genetic resources including the completely sequenced and annotated genome of *M. guttatus* (www.phytozome.net, Wu et al 2007). Like *Stephanomaria*, it is an excellent system for the study of local geographic speciation because it consists of the large-ranged outcrossing putative progenitor species *M. guttatus,* and many geographically restricted

morphological species that are often self-fertilizing and adapted to specialized edaphic environments (Wu et al 2007). The evolutionary history of the species complex is largely unresolved due to recent divergence and ongoing interspecific introgression. Recently a new morphological species has been described in the complex, *Mimulus filicifolius* (Sexton et al 2013). *M. filicifolius* was originally categorized as a geographically disjunct population of the *M. guttatus's* close relative *Mimulus laciniatus. M. laciniatus* is a highly self-fertilizing annual that occurs from three to nine thousand feet in the central and southern Sierra Nevada Mountains of California, USA (Figure 4). Both *M. laciniatus* and *M. filicifolius* have highly lobed leaves, small flowers, and are endemic to similar dry, exposed granite outcrop habitat. They are the only species with dissected leaves in the entire genus *Mimulus. M. filicifolius* was described as a new species due primarily to its more finely divided bi-pinnately compound leaves. *M. filicifolius* is restricted to a few populations in eastern Butte and western Plumas Counties in the northern foothills of the Sierra Nevada around one thousand feet in elevation and over 100 miles away from any known *M. laciniatus* population (Figure 4, Sexton et al 2013). However it is not known whether *M. filicifolius* is genetically distinct and/or reproductively isolated from *M. laciniatus,* or if it is simply a morphologically divergent variety of *M. laciniatus*.

To address this question we examined patterns of genetic variation in four members of the *M. guttatus* species complex: *M. guttatus, M. nasutus, M. laciniatus* and *M.*

filicifolius. M. guttatus is a genetically diverse outcrossing species that occurs in moist seeps and streams across much of western North America (Figure 4). *M. nasutus* is a highly self-fertilizing species that also lives in seeps and streambeds primarily along the west coast of North America from British Columbia to northern Mexico. These two closely related species' have overlapping geographic ranges that encompass the geographic range of both *M. laciniatus* and *M. filicifolius* (Figure 4, Sweigart and Willis 2003, Modliszewski and Willis 2012, Brandvain et al in review). We included *M. guttatus* and *M. nasutus* in this analysis to see whether *M. filicifolius* was more genetically similar to *Mimulus* species in close geographic proximity than to *M. laciniatus*. In this study we use a combination of ecological measurements, molecular population genetics, flow cytometry, and interspecific crosses to address four main questions: *1)* Does the recently described *M. filicifolius* differ from *M. laciniatus* in ecology, mating system, or genome size as well as in morphology? *2)* Is *M. filicifolius* genetically distinct from *M. laciniatus*? 3) Is *M. filicifolius* reproductively isolated from *M. laciniatus* or other members of the *M. guttatus* species complex?

2.3 Materials & Methods

2.3.1 Habitat Characterization & Collections of four *Mimulus* species

Although *M. guttatus, M. nasutus, M. laciniatus,* and *M. filicifolius* overlap a great deal in their geographic ranges they seem to occupy very distinct habitats within those

Table 3. Population location and sequencing information for all individuals used in genetic principal components analysis

Population	*Species*	Locale	Longitude	Latitude	Elevation	No. CYCA	No. Mg1	No. Mg2	No. Mg3	No. Mg4	No. Mg5	No. Mg6
Mden	*M. dentilobus*	-	-	-	-	-	-	-	-	1	-	-
BR	*M. filicifolius*	Bald Rock, Butte Co., CA	-121.34	39.6445	3020 ft	1	1	1	1	1	1	1
Mgv	*M. glabratus var. fremontii*	Catron Co., NM	-108.85	33.631	-	-	1	1	1	-	1	1
ANR	*M. guttatus*	Angelo Reserve, CA	-123.63	39.737	1348 ft	1	1	1	-	1	1	-
BCB	*M. guttatus*	Big Creek Beach, Monterey Co., CA	-121.36	36.0377	15 ft	1	-	-	-	-	-	-
BVG	*M. guttatus*	Ben Irving Reservoire, OR	-123.57	43.059	957 ft	-	1	1	-	2	2	2
CEC	*M. guttatus*	Central Camp Road, Fresno Co., CA	-119.3	37.1852	4182 ft	-	1	-	-	-	1	1
DAV	*M. guttatus*	Davenport Beach, Santa Cruz Co., CA	-122.13	37.015	15	3	-	-	-	-	-	-
DCR	*M. guttatus*	Deer Creek Rd., Tehama Co., CA	-	-	-	2	-	-	-	-	-	-
DRG	*M. guttatus*	Dexter's Reservoire, OR	-122.76	43.917	898 ft	-	2	3	3	3	3	3
DUN	*M. guttatus*	Oregon Dunes Natl. Rec. Area, Lane Co., OR,	-	-	-	1	-	-	-	-	-	-
FAL	*M. guttatus*	Fales hot springs, Mono Co., CA	-	-	-	-	-	1	-	-	-	-
GBC	*M. guttatus*	Boulder Creek, Santa Cruz Co., CA	-	-	-	1	-	-	-	-	-	-
GCC	*M. guttatus*	Chinese Camp, Tuolumne Co., CA	-	-	-	-	-	1	-	-	-	-
GHP	*M. guttatus*	Galice-Hellgate pulloff, Josephine Co., OR	-123.31	42.3293	744ft	1	2	2	-	-	-	-
GOS	*M. guttatus*	Occidental Rd., Sonoma Co., CA	-	-	-	-	-	-	-	-	-	-
GRE	*M. guttatus*	Greyhound Rock, Santa Cruz Co., CA	-	-	-	1	-	-	-	-	-	-
GTR	*M. guttatus*	Berryessa Knoxville Rd., Napa Co., CA	-	-	-	-	1	1	-	-	-	-
HBG	*M. guttatus*	Howard Buford Recreation Area, Lane Co. OR	-122.58	44.0023	774 ft	-	1	2	-	-	-	-
HCG	*M. guttatus*	Hog Creek, OR	-123.5	42.54	800 ft	-	4	3	2	2	1	2
HEC	*M. guttatus*	Heceta Beach, Lane Co., OR	-124.07	44.081	15	1	-	-	-	-	-	-
IM	*M. guttatus*	Iron Mountain, Linn Co., OR	-	-	-	-	1	1	1	-	1	1
KCH	*M. guttatus*	Kings Canyon Highway, CA, USA	-119.01	36.4402	4877 ft	-	11	-	-	-	1	1
LMC	*M. guttatus*	Lower Mendocino County, CA	-123.05	38.5184	1004	1	-	-	-	-	-	-
MAR	*M. guttatus*	Near Oakland, OR	-123.29	43.479	590 ft	-	4	5	2	3	3	3
MID	*M. guttatus*	Middlefork, Tuolumne Co., CA	-	-	-	-	-	1	-	-	-	-
MPL	*M. guttatus*	Mapleton, Lane Co., OR	-	-	-	1	-	-	-	-	-	-
NDR	*M. guttatus*	Dos Rios, CA	-123.35	39.708	-	1	-	1	-	1	1	-
NFG	*M. guttatus*	North Fork Reservoire, OR	-122.25	45.234	-	-	3	3	3	2	-	3
NHG	*M. guttatus*	Nanoose Hill, VI, BC, CAN	-124.16	49.273	-	-	2	1	-	2	2	2
NKL	*M. guttatus*	Nimpkish Lake, VI, BC, CAN	-126.93	50.358	357	-	1	1	1	1	1	1

Population	*Species*	Locale	Longitude	Latitude	Elevation	No. CYCA	No. Mg1	No. Mg2	No. Mg3	No. Mg4	No. Mg5	No. Mg6
OPB	*M. guttatus*	Otter Point State Park, Curry Co. OR	-124.25	42.2784	25	1	-	-	-	-	-	-
OSW	*M. guttatus*	Oswald West State Park, Tillamook Co. OR	-123.58	45.4567	15	2	-	-	-	-	-	-
PTH	*M. guttatus*	Poopenaough Trail Head, Yosemite NP, CA	-119.49	37.545	4642 ft	-	1	-	-	-	1	1
PTR	*M. guttatus*	Pt Reyes, Marin Co., CA	-	-	-	2	-	-	-	-	-	-
QCI	*M. guttatus (4n)*	Queen Charlotte Islands, BC, CAN	-	-	-	-	-	-	-	1	1	1
RGR	*M. guttatus*	Rogue River, Curry Co. OR	-124.13	42.2936	270	3	-	-	-	-	-	-
ROG	*M. guttatus*	12 mi Southwest of Marial, OR	-123.64	42.657	-	-	-	3	2	3	3	3
S2G	*M. guttatus*	Spencer's Butte, Lane Co., OR	-123.09	43.9822	1715 ft	-	-	1	1	1	1	1
SAG	*M. guttatus*	Near Mehama, OR	-122.54	44.797	-	-	1	2	-	2	2	2
SAM	*M. guttatus*	Saddle Mountain, Clatstop Co., OR	-123.41	45.5756	1938	2	-	-	-	-	-	-
SBG	*M. guttatus*	Sherar's Bridge, OR	-121.04	45.251	-	-	1	2	-	-	-	-
SHL	*M. guttatus*	Shaver Lake, Fresno Co., CA	-119.18	37.0868	5231 ft	-	1	-	-	-	1	1
SIM	*M. guttatus*	Simpson Beach, Coos Co., OR	-	-	-	1	-	-	-	-	-	-
SKZ	*M. guttatus*	Skutz Falls, VI, BC, CAN	-123.95	48.783	382	-	2	2	2	1	2	2
SPO	*M. guttatus*	Shelter Point, Comox-Strathcoma Co., BC, CAN	-125.11	49.5479	-	1	-	-	-	-	-	-
SSG	*M. guttatus*	Salt Spring Island, BC, CAN	-123.43	48.739	-	-	1	1	-	2	2	2
SWB	*M. guttatus*	Spearm Whale Beach, Mendocino Co., CA	-123.41	39.0216	15	1	-	-	-	-	-	-
SWC	*M. guttatus*	Sweet Creek Road, Lane Co., OR	-123.54	43.5757	330	1	-	-	-	-	-	-
TAY	*M. guttatus*	Taylor River Rest Stop, VI, BC, CAN	-125.29	49.299	196	-	2	2	2	2	2	2
TRU	*M. guttatus*	Hwy. 89, Truckee, Placer Co., CA	-	-	-	-	1	1	-	-	-	-
USB	*M. guttatus*	Usal Beach, Mendocino Co., CA	-123.51	39.4993	15	1	-	-	-	-	-	-
VoR	*M. guttatus*	Valley of the Rogue State Park, OR	-123.13	42.414	971 ft	-	-	2	-	-	-	-
YVO	*M. guttatus*	Yosemite Valley Overlook, Yosemite NP, CA	-119.45	37.434	5101 ft	1	-	-	1	1	-	-
BP	*M. lacinaitus*	Bad Point, Sierra National Forest, CA	-119.26	37.238	8000 ft	1	1	1	1	1	1	1
DNK	*M. laciniatus*	Dinky Creek, Sierra National Forest, CA	-119.13	37.0511	6075 ft	1	1	1	1	1	1	1
GB	*M. laciniatus*	Grand Bluff, Sierra National Forest, CA	-119.14	37.0425	5510 ft	1	1	1	1	1	1	1
LIB	*M. laciniatus*	Little Baldy, King's Canyon NP, CA	-118.48	36.3696	7206 ft	1	1	1	1	1	1	1
ST	*M. laciniatus*	Snow Trail, Yosemite NP, CA	-119.54	37.7664	6135 ft	1	1	1	1	1	1	1
TIG	*M. laciniatus*	Tiago Road, Yosemite NP, CA	-119.3	37.4872	8489 ft	1	1	1	1	1	1	1
WLF	*M. laciniatus*	White Wolf, Yosemite NP, CA	-119.36	37.5049	7859 ft	1	1	1	1	1	1	1
WUV	*M. laciniatus*	Wuksaki Village, Kings Canyon NP, CA	-118.45	36.3629	6987 ft	1	1	1	1	1	-	1

Population	*Species*	Locale	Longitude	Latitude	Elevation	No. CYCA	No. Mg1	No. Mg2	No. Mg3	No. Mg4	No. Mg5	No. Mg6
BRI	*M. nasutus*	Bridal Veil Falls, Mariposa Co., CA	-	-	-	2	-	-	-	-	-	-
CHV	*M. nasutus*	Chivo Falls, Sahuaro NP, Pima Co., AZ	-	-	-	1	-	-	-	-	-	-
CLR	*M. nasutus*	Columbia River, Klickitat Co., WA	-	-	-	1	-	-	-	-	-	-
CMF	*M. nasutus*	Camp Meeker Falls, CA	-122.97	38.443	236	-	1	1	-	1	1	-
FGC	*M. nasutus*	Fern Glen Canyon, Grand Canyon, AZ	-	-	-	1	-	-	-	-	-	-
HCN	*M. nasutus*	Hog Creek, OR	-123.5	42.54	800 ft	-	1	1	1	1	1	1
KIN	*M. nasutus*	Hwy 180 near King's Canyon, Fresno Co., CA	-	-	-	2	-	-	-	-	-	-
KRR	*M. nasutus*	King's Ridge Road, CA	-123.12	38.58	434	-	1	1	-	1	1	-
M12	*M. nasutus*	Dear Creek Rd. mi. 12, Tahama Co., CA	-	-	-	1	-	-	-	-	-	-
MEN	*M. nasutus*	Jacksonville Rd. jct., Tuolumne Co., CA	-	-	-	1	1	1	-	1	1	-
MHA	*M. nasutus*	Mt. Hamilton, CA	-121.66	37.336	3157	-	1	1	-	1	1	-
NBC	*M. nasutus*	Boulder Creek, CA	-122.12	37.13	-	1	1	1	-	1	1	-
NCL	*M. nasutus*	Cherry Lake Rd., Tuolumne Co., CA	-	-	-	1	-	-	-	-	-	-
NDP	*M. nasutus*	Don Pedro Vista Point, Tuolumne Co., CA	-	-	-	2	-	-	-	-	-	-
NFN	*M. nasutus*	North Fork Reservoire, OR	-122.25	45.234	-	-	1	1	1	1	1	1
NHN	*M. nasutus*	Nanoose Hill, VI, BC, CAN	-124.16	49.273	-	-	1	1	-	1	1	-
NMD	*M. nasutus*	Monticello Dam, Solano Co., CA	-	-	-	4	-	-	-	-	-	-
SBN	*M. nasutus*	Sherar's Bridge, OR	-121.04	45.251	-	-	-	1	-	1	1	-
SF	*M. nasutus*	Sherar's Falls, OR	-121.04	45.26	-	2	1	1	1	1	1	1
SHI	*M. nasutus*	Standish-Hickey State Recreation Area, CA	-	-	-	-	1	1	-	1	1	-
SNF	*M. nasutus*	Sierra National Forest, Fresno Co., CA	-	-	-	1	-	-	-	-	-	-
TOK	*M. nasutus*	Tokopah Falls, Sequoia NP, Tulare Co., CA	-	-	-	2	-	-	-	-	-	-
TRT	*M. nasutus*	near Troutdale, OR	-122.37	45.52	-	-	1	1	-	1	1	-
WSK	*M. nasutus*	White Salmon River, Klickitat Co., WA	-121.52	45.736	-	-	3	2	-	3	3	2
MpPLA	M. platycalyx	Umpqua River, Douglas Co., OR	-	-	-	1	-	-	-	-	-	-
KNR	*M. unknown*	Kneeland Road, Humbolt Co., CA	-123.56	40.4042	2154	-	-	2	-	-	-	-
Myec	*M. yecorensis*	Yecora, Sonora Mexico	-	-	-	1	-	-	-	-	-	-

ranges. To quantitatively characterize each species' habitat we measured three environmental variables (percent soil moisture, soil saturation point, and ground temperature) in four populations of *M. guttatus,* two of *M. nasutus,* seven of *M. laciniatus,* and one of *M. filicifolius.* We chose to measure these three variables because they should capture much of the abiotic environmental variation between rocky outcrop and grassy seep habitats. Soil moisture and soil saturation point were measured with a Decagon soil moisture probe while ground temperature was measured using an infrared thermometer.

Environmental variables were measured across 3-5 transects per habitat type per population site. We chose nine population sites that differed in elevation and geographic location: Bald Rock (*M. filicifolius*), Sandy Bluff (*M. laciniatus* & *M. guttatus*), Cedar Vista (*M. guttatus*), Peterson Road (*M. laciniatus, M. nasutus,* & *M. guttatus*), Willow Creek (*M. laciniatus, M. nasutus,* & *M. guttatus*), Central Camp Road (*M. laciniatus*), Dinky Creek(*M. laciniatus* & *M. guttatus*), Shaver Lake (*M. laciniatus* & *M. guttatus*), and Huntington Lake (*M. laciniatus*). All measurements took place between May10th and May 30th, 2010. Each population was surveyed multiple times over the twenty days at different times of day. To describe the ecological variation between species we performed a principle components analysis (PCA) on our three five environmental variables measured in all population sites. In order to test whether

species habitats differed significantly in these three variables we performed three one-way analyses of variance with each environmental variable as the dependent variable and species as the independent variable. In order to control for repeated measures we averaged the our environmental measurements at each point in each transect across time. To examine which species differed significantly in each variable we performed Tukey HSD tests on each of our one-way ANOVA's.

2.3.2 Comparing the Mating System and Genome Size of *M. filicifolius* and *M. laciniatus*

We used the populations described in Table 5 to characterize the mating system and genome size of the new granite outcrop species *M. filicifolius* for comparison to *M. laciniatus.* To characterize the mating system of *M. filicifolius,* we genotyped individuals in the Bald Rock population of *M. filicifolius* and five *M. laciniatus* populations at 11 co-dominant markers. We used three single-copy, nuclear-gene-intron-length markers (Fishman and Willis 2005; Sweigart et al. 2006; Lowry et al. 2008) and eight microsatellite markers (Kelly and Willis 1998). Marker sequences are listed in Table S4 of Sexton et al. (2011). PCR products were analyzed with an ABI 3730 DNA Analyzer and size polymorphisms were visually scored in GENEMARKER (SoftGenetics LLC, State College, PA). All markers were located on different linkage groups and therefore represent genetically independent loci. Population genetic estimates were averaged across loci for all populations (Table 6). We used GenAlEx version 6 (Peakall and

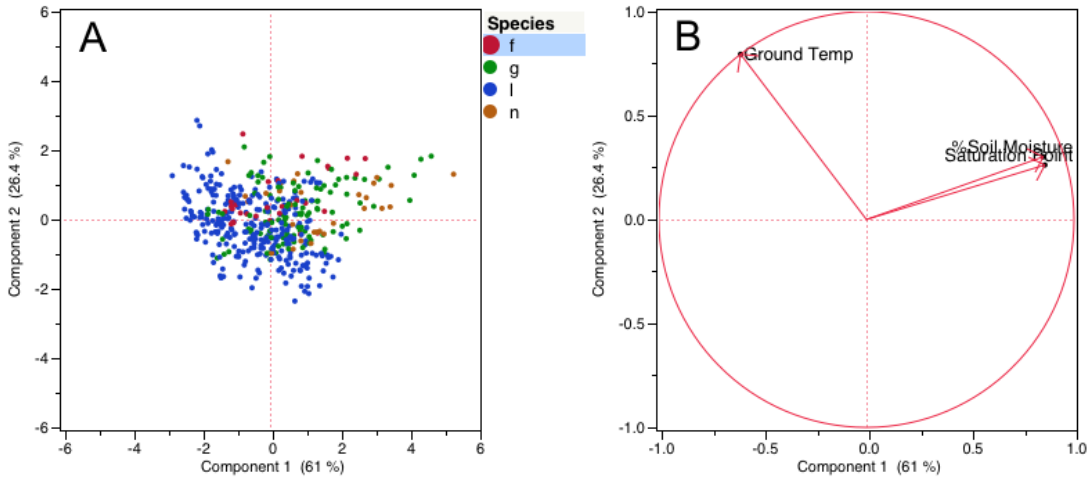

Figure 5. Principal components analysis of three environmental variables (soil moisture, soil saturation point, and ground temperature) measure across the habitats of *M. filicifolius* (red), *M. laciniatus* (blue), *M. nasutus* (orange), and *M. guttatus* (green). A) Is a plot of pc scores for each species along PC1 and PC2, B) Is a loading plot indicating the how the original variables change along the PC1 and PC2. The direction of the arrow indicates that whether a variable is increasing of decreasing.

Smouse 2006) to calculate population genetics estimates (F statistics) and to test for Hardy-Weinberg equilibrium within populations. We obtained genome size estimates of *M. filicifolius* from the Bald Rock population (Sexton et al. 2013) and *M. laciniatus* from two populations approximately 60 km apart: Devil's Postpile National Monument and Grand Bluff in the Sierra National Forest. For each population, approximately 30 seeds from a single maternal family of one field collected plant were pooled for flow cytometry (FCM) analysis with three (much larger) seeds of an internal standard, *Solanum lycopersicum*, having a diploid (2C) genome size of 1.96 pg (Doležel et al. 1992). Approximate genome size was then estimated through comparison with the internal

Table 4. Results of a one-way analysis of variance with each environmental variable as the dependent variable and species as the independent variable. We report the degrees of freedom (Df), Sum of Squares (Sum Sq), Mean Square (Mean Sq), F-statistic (F-value), and p-value from each ANOVA.

Environmental Variable	Model	Df	Sum Sq	Mean Sq	F-value	p-value
% Soil Moisture	Species	3	8882	2960.8	42.88	<0.0001
	Residual	456	31499	69.1		
Soil Saturation Point	Species	3	16801	5600	48.72	<0.0001
	Residual	456	52413	115		
Ground Temperature	Species	3	785	261.7	4.29	0.0053
	Residual	456	27809	60.99		

standard by the general relationship: genome size (bp) = (0.978 X 10^9) X DNA content (pg) (Doležel et al. 2003). Additionally, *M. filicifolius* seeds were pooled with the Grand Bluff population of *M. laciniatus* seeds and analyzed using FCM to verify genome size differences through analysis of double-peaks (Smarda et al. 2008). Sample and solution preparation and FCM analyses were performed as in McIntyre (2012) at University of California, Davis. FCM data were visualized using Cyflogic data analysis tool (version 1.2.1; http://www.cyflogic.com/).

2.3.3 Is *M. filicifolius* genetically distinct from *M. laciniatus*?

In order to determine whether *M. filicifolius* is a genetically distinct species we sampled populations across the geographic ranges of *M. guttatus*, *M. nasutus*, *M. laciniatus*, and *M. filicifolius*. We collected between 15 and 50 individuals per population from 8 *M. laciniatus*, 5 *M. guttatus*, and 1 *M. filicifolius* population(s) across the Sierra Nevada

Table 5. Results of TukeyHSD test of the significance of difference between species means for % Soil Moisture, Soil Saturation Point, and Ground Tmperature. significance level denoted by this code: p-value <0.05*, <0.01,0.001*****

Species Contrast	Diff % Soil Moisture	Diff Soil Sat Point	Diff Ground Temp
M. gutt vs. M. fili	3.90	0.12	-4.93*
M. lac vs. M. fili	-5.02**	-9.92***	-3.25
M. nas vs. M. fili	6.77**	9.5**	-5.95*
M. lac vs. M. gutt	-8.92***	-10.04***	1.68
M. nas vs. M. gutt	2.86	9.38***	-1.02
M. nas vs. M. lac	11.79***	19.42***	-2.70

mountain range (Table 3). All 30 *M. nasutus* and the other 47 *M. guttatus* populations used in this analysis are from previous collections (Sweigart and Willis 2003, Modliszewski and Willis 2012). When possible we collected sympatric population pairs. Samples were derived from live plants that were propagated in the greenhouse and self-fertilized to produce seed. The progeny of these seed families were grown in the greenhouse under uniform conditions where tissue was collected for DNA extraction. Three of our *M. laciniatus* populations (GB, BP, & ST) and the Bald Rock (BR) population of *M. filicifolius* were used in both sequencing and microsatellite analyses. *M. nasutus* and *M. guttatus* populations were only used in our sequencing analysis.

To examine patterns of genetic variation in these four species portions of 7 nuclear genes were amplified in populations from across the range of *M. guttatus, M. nasutus*, *M. laciniatus*, and *M. filicifolius* by polymerase chain reaction (PCR). Genomic

DNA was isolated from leaf and bud tissue using a modified CTAB extraction protocol (Kelly and Willis 1998). Primers for the CYCLOIDEA-A (CYCA) locus were developed from a portion of the gene's first exon (Sweigart and Willis 2003). The other six loci, *Mg1-6,* were developed from exonic regions of single copy genes. They were chosen because they represent the average sequence divergence between *M. nasutus* SF and *M. guttatus* IM62 (Modliszewksi and Willis 2012). We used already published sequence data from 30 *M. nasutus* and 47 *M. guttatus* individuals (Sweigart and Willis 2003, Modliszewski and Willis 2012) and added sequences from our 8 *M. laciniatus*, 5 *M. guttatus*, and single *M. filicifolius* populations. PCR conditions, primers, and all available sequence data can be found in Modliszewski and Willis (2012).

M. laciniatus is a highly self-fertilizing species and therefore we expect the majority of its genome to be homozygous (Adawalla and Ritland 1997). Consequently a single individual from each *M. laciniatus* population was directly sequenced at each of the above nuclear loci. Since *M. guttatus* is a genetically diverse outcrossing species we expected it to be heterozygous at many of these loci. All *M. guttatus* individuals were first directly sequenced, but then PCR products were cloned into the pGEM® –T Easy Vector system. Six colonies from each individual were PCR amplified and direct sequenced to identify both alleles at each locus and check for PCR -generated errors such as point mutations or recombination. DNA sequences from each of the seven loci

were aligned in Sequencher (Gene Codes Corp., Anne Arbor, MI, USA). Chromatograms were used to identify and correct erroneous polymorphism. Ambiguous insertion/deletion polymorphisms were removed from the data set by eye using MacClade (© 2011 David R. and Wayne P. Maddison). Basic polymorphism data was obtained using the program DNAsp (Rozas and Rozas 1999).

To investigate the degree of genetic similarity across species we performed principal components analysis (PCA) on our sequence data using the R package adegenet 1.3-8 (Jombart 2008). This is a distance-based method that clusters DNA sequences based on their genetic similarity. We performed two genetic principle components analysis: one on the CYCA locus and one on a concatenated alignment combining the six *M. nasutus* based EST loci (*Mg1-6*). The CYCA locus was left out of the concatenated analysis because it did not contain enough overlapping individuals with the *Mg* alignment. The *M. guttatus* and *M. nasutus* population dataset for the CYCA locus does not overlap very much with that of the *Mg1-6* loci since the former was generated by Sweigart and Willis (2003) and the latter generated by Modliszewski and Willis (2012). Including the CYCA data allows us to compare the patterns of genetic variation between two independent sets of populations from the same four species. We computed 95% confidence intervals (95% CI) for the mean genetic value of *M. guttatus*, *M. nasutus*, and *M. laciniatus* along all three major principal component (PC) axes to

determine their genetic similarity. Since we only had a single sequence of *M. filicifolius* in our analysis we could not compute confidence intervals for its PC values. In order to determine whether *M. filicifolius* was genetically distinct from the other three species we compared its location along each PC axis to the 95% CI's of the other three species.

2.3.4 Is there post-zygotic reproductive isolation between *M. filicifolius* and the *M. guttatus* species complex?

In order to ascertain whether post-zygotic reproductive isolation existed between the new species *M. filicifolius* and members of the *M. guttatus* species complex crosses were made between inbred lines of *M. laciniatus* & *M. filicifolius*, and *M. guttatus* & *M. filicifolius*. In each cross F1 hybrids were self-fertilized either by hand or automatically because the cross was between two self-fertilizing taxa. Hybrid fertility was assessed in each cross with *M. filicifolius* by counting the number of fruits containing viable seeds that developed post self-fertilization.

2.4 Results

2.4.1 Do species with overlapping ranges have quantitatively different niches?

To better understand the ecological divergence or convergence of these species we characterized the habitat of each one. We used principal components analysis (PCA) to graphically describe the environmental variation in percent soil moisture, soil saturation point, and ground temperature across the four species habitats (Figure 5). The

Table 6. Population location and genetic summary statistics for 11 loci for *Mimulus filicifolius* and *M. laciniatus* collection sites in the California Sierra Nevada. Population genetic statistics include the number samples per site (*N*), observed heterozygocity (H_o), expected heterozygocity (H_e), mean fixation index (*F*), and the rate of self-fertilization (*S*).

Site	*N*	Latitude	Longitude	*F*	H_e	H_o	*S*
Mimulus filicifolius							
BR	28	39.6445	-121.343	0.93	0.59	0.04	0.963731
Mimulus laciniatus							
GB	33	37.0746	-119.23	0.86	0.76	0.11	0.924731
BP	46	37.2384	-119.26	0.95	0.63	0.03	0.974359
JM	41	37.5069	-119.339	0.91	0.63	0.06	0.95288
HS	43	37.8939	-119.849	0.92	0.73	0.07	0.958333
ST	46	37.7663	-119.542	0.88	0.73	0.09	0.93617
Mean	**41.8**	**-**	**-**	**0.9**	**0.72**	**0.07**	**0.94929**

majority of the variation in habitat type is explained by the first and second principal components (PC1=61%, PC2=26.4%). As PC1 increases soil moisture increases and ground temperature decreases (Figure 5). It appears that *M. nasutus* and *M. guttatus* have largely overlapping habitats that are higher in soil moisture and lower in ground temperature than *M. laciniatus (*Figure 5). This is consistent with our expectations. In our PC scores plot it appears that the habitat of *M. filicifolius*, the newly described granite outcrop endemic in the genus, overlaps with that of *M. laciniatus* more so than either *M. nasutus* or *M. guttatus* (Figure 5).

To test whether these species' habitats differed significantly in soil moisture and ground temperature we performed three one-way ANOVA's. We found that species differed significantly in percent soil moisture, soil saturation point, and ground temperature (Table 4). Our post hoc comparisons using the Tukey HSD test indicate that

M. nasutus and *M. guttatus* occur in habitats with similar ground temperatures (means = 16.6°C, 17.6°C) and levels of soil moisture (means = 25.2%, 22.3% soil moisture, Table 5). *M. laciniatus's* habitat is warmer than *M. guttatus* and *M. nasutus'* (mean = 19.3°C) and significantly drier than all three other species (mean = 13.4% soil moisture, Table 5). *M. filicifolius'* habitat is similar to *M. laciniatus's* in ground temperature (mean = 22.5°C) and is significantly warmer than *M. nasutus* and *M. guttatus'* and drier than *M. nasutus'* (mean = 18.4% soil moisture, Table 5). Thus we see that overall *M. filicifolius* and *M. laciniatus's* granite outcrops are significantly warmer and drier than *M. guttatus* and *M. nasutus's* seeps.

2.4.2 Does *M. filicifolius* differ from *M. laciniatus* in mating system or genome size?

In our mating system characterization of *M. filicifolius* and *M. laciniatus* we found that all genetic markers successfully amplified across populations and were highly polymorphic. Marker scores were consistent across repeatability tests. Estimates of selfing rates based on genetic markers were similar between the two species and indicate that both species are highly self-fertilizing. As expected for self-fertilizing species, all loci in all *M. filicifolius* and *M. laciniatus* populations were not at Hardy-Weinberg equilibrium ($P < 0.001$). The fixation index (F) for *M. filicifolius* was 0.93 and the mean fixation index for the five *M. laciniatus* localities was 0.90 (Table 6). We used the

Table 7. Sequence polymorphism statistics for each locus and species: S stands for the number of segregating sites and pi is the estimate of 4Nu using average number of nucleotide differences per site.

Locus	Size(bp)	S M.laciniatus	S M.nasutus	S M.guttatus	S M.filicifolius	π M.laciniatus	π M.nasutus	π M.guttatus	π M.filicifolius
CYCA	391	2	9	22	NA	0.00307	0.00375	0.00731	NA
Mg1	367	6	0	13	NA	0.00409	0	0.01083	NA
Mg2	287	3	0	19	NA	0.00261	0.00131	0.01704	NA
Mg4	408	1	0	10	NA	0.00131	0	0.00483	NA
Mg5	457	6	2	41	NA	0.00458	0.00088	0.02032	NA
Mg6	499	11	8	45	NA	0.00587	0.0029	0.01782	NA

equation S=2F/(1+F) to calculate S, the rate of self-fertilization, in each population (Hartl and Clark 1997). One caveat is that this estimate of S assumes that it is at equilibrium and this may not in fact be the case. The rate of self-fertilization (***S)*** for *M. filicifolius* was 0.96, which is similar to the average ***S*** of our *M. laciniatus* populations, 0.95 (Table 6). *M. laciniatus* and *M. filicifolius* have similarly high levels of inbreeding and self-fertilization indicating that they have similar mating systems.

To assess whether the newly described species *M. filicifolius* was divergent in overall genome size we performed flow cytometry analysis on a cohort of *M. laciniatus* populations and the Bald Rock population of *M. filicifolius*. The sampled *M. filicifolius* family had a mean genome size estimate of 0.65pg or 315 Mb (CV = 1.41%), whereas the *M. laciniatus* samples averaged 0.72 pg or 360 Mb (CV = 2.04%) for the Devils Postpile population using the internal standard; and 0.85 pg or 367 Mb (CV = 0.79%) for the Grand Bluff population (estimated from double-peak analysis). Our genome sizes are

smaller than those estimated for close relatives *M. guttatus* and *M. nasutus* by Modliszewski and Willis (2012) however this could be due to the fact that they used petunia as a genome size standard while we used tomato. From these estimates genome size differs in the range of 10-14% between *M. filicifolius* and *M. laciniatus* and ca. 4% between the two *M. laciniatus* populations sampled.

2.4.3 Determining whether *M. filicifolius* is a genetically distinct species

The loci used in this analysis showed varying amounts of genetic diversity across *Mimulus* species. *CYCA*, *Mg5*, and *Mg6*, had the greatest levels of informative genetic variation while *Mg1* had the least (Table 7). The greatest proportion of molecular variation at each locus was explained by within *M. guttatus* polymorphism. To determine whether *M. filicifolius* is genetically as well as morphologically divergent, we performed genetic principal components analysis (PCA) at *CYCA* and our six *Mg* nuclear loci (Modliszewski and Willis 2012). We found several interesting patterns.

In the *CYCA* analysis PCs 1, 2 & 3 explained 30.7, 12.4, and 10.5 percent of the genetic variance respectively while in the concatenated *Mg* loci analysis PC's 1, 2, &3 explained 9.6, 5.7, & 5.3 percent of the genetic variance. The 95% confidence intervals (95% CI) for the mean *M. laciniatus* and *M. guttatus* genetic values were non-overlapping for PC1 & PC2 in the concatenated *Mg* PCA, but only for PC1 in the *CYCA* analysis. The

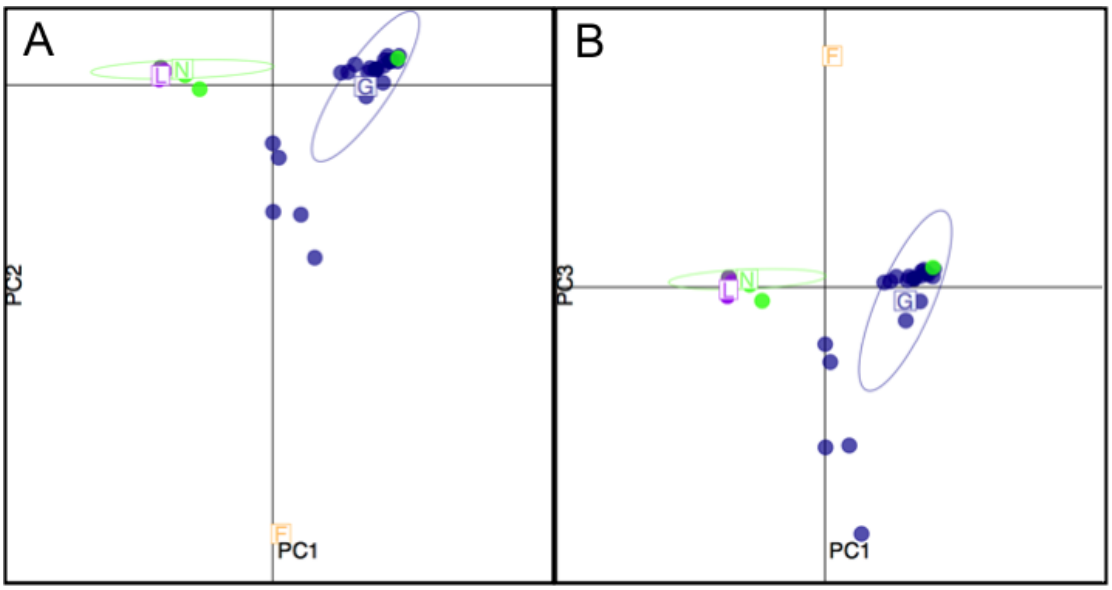

Figure 6. Principal components analysis (PCA) of *M. guttatus* (red), *M. nasutus* (blue), *M. laciniatus* (green), and *M. filicifolius* (purple) genetic diversity at the CYCA locus. A) A plot of genetic pc scores at the CYCA locus along axes PC1 and PC2. B) A plot of genetic pc scores at the CYCA locus along axes PC1 and PC3.

degree of genetic similarity between *M. guttatus* and *M. nasutus* follows a similar pattern with non-overlapping 95% CI's for mean genetic values in PCs' 1 & 2 in the *Mg* loci and only for PC1 at *CYCA (*Table 8). This indicates that both *M. laciniatus* and *M. nasutus* are significantly genetically differentiated from *M. guttatus* along the principal components explaining the greatest amount of genetic variation, but that both species are still genetically similar to *M. guttatus* as expected for recently diverged and potentially hybridizing taxa. For *M. laciniatus* and *M. nasutus* the *CYCA* and *Mg* loci show very different patterns of genetic similarity. At the *CYCA* locus the 95% CI's for the mean *M. laciniatus* and *M. nasutus* genetic values are highly overlapping at PC1, PC2, and PC3

(Table 8). This pattern of genetic overlap between these two species is apparent in our PCA plots at *CYCA* (Figure 6). However we do not see this pattern in our concatenated *Mg* loci analysis (Figure 7). At the *Mg* loci none of the mean PC's have overlapping 95% CI's between *M. laciniatus* and *M. nasutus,* although the confidence intervals are close at PC1 (Table 8). Therefore at the majority of genetic loci *M. nasutus* and *M. laciniatus* are genetically distinct.

In both our PCA's, at the CYCA locus and the consensus sequence of the *Mg* loci, *M. filicifolius* is clearly genetically divergent from *M. laciniatus* in terms of the first three principal components (Figures 6 & 7). In fact *M. filicifolius* does not cluster with any of the other three species, but remains genetically distinct from *M. laciniatus*, *M. nasutus,* and *M. guttatus* in all analyses (Figures 6 & 7). Our single *M. filicifolius* sample is outside the 95% confidence intervals (95% CI) of *M. guttatus, M. nasutus,* and *M. laciniatus* at PC's 1 through 3 (Table 8). *M. filicifolius* is particularly divergent from *M. laciniatus* by being more than 3 standard deviations away from the mean *M. laciniatus* value for PC's 1, 2, & 3. It is not clear from this analysis what species *M. filicifolius* is most closely related to, but it is most certainly genetically distinct from *M. laciniatus* despite its similar habitat, level of self-fertilization, and lobed leaf shape.

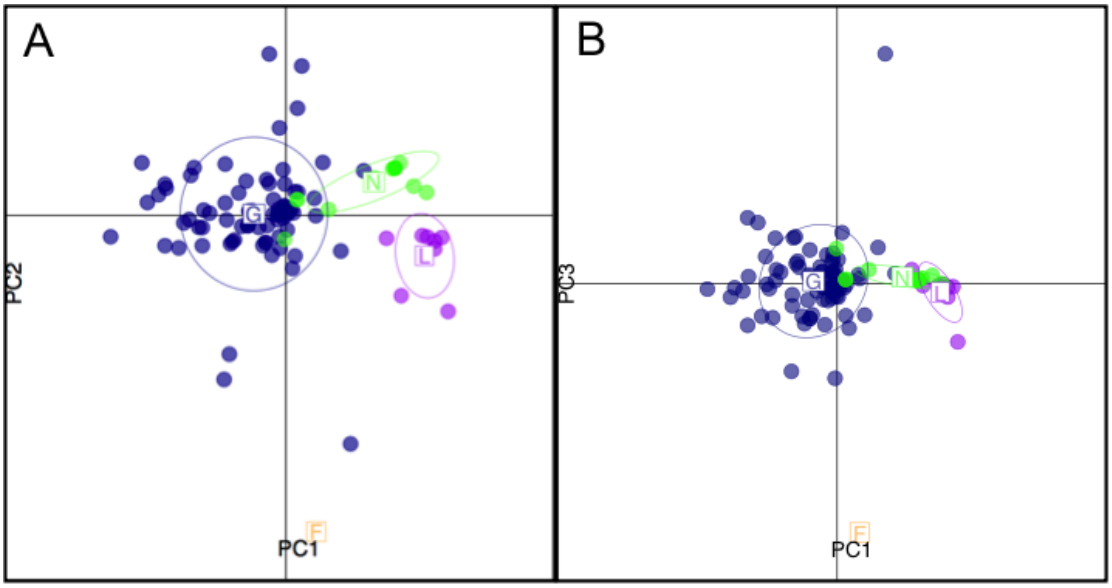

Figure 7. Principal components analysis (PCA) of *M. guttatus* (red), *M. nasutus* (purple), *M. laciniatus* (green), and *M. filicifolius* (blue) genetic diversity on a concatenated alignment of six nasutus based EST loci, *Mg1-6*. A) A plot of genetic pc scores for the concatenated *Mg* loci along axes PC1 and PC2. B) A plot of genetic pc scores for the concatenated *Mg* loci along axes PC1 and PC3.

2.4.4 Is *M. filicifolius* reproductively isolated?

To ascertain whether post-zygotic reproductive isolation existed between *M. filicifolius* and other members of the species complex we crossed this species to *M. laciniatus* and *M. guttatus.* We found that both crosses produced viable F1 hybrids. However, in our *M. laciniatus x M. filicifolius* F1's we found no fruit with viable seeds out of hundreds of autonomously self-pollinated ovules. This was unexpected since the species have similarly sized flowers and both self-fertilize automatically before flowers even open. *M. laciniatus x M. filicifolius* F1 flowers had no obviously morphological

defects. Many fruits developed, but they were empty. Similarly when *M. guttatus* x *M. filicifolius* F1's were self-fertilized barely any seed was produced by either hand or autonomous self-pollination (1 out of 36 hand pollinations, 0 autonomous pollinations). For comparison, when *M. laciniatus x M. guttatus* F1's were self-fertilized in a separate quantitative trait locus mapping experiment a large viable F2 population was created (thousands of seeds, 700 were planted and germinated successfully). This is evidence of a strong hybrid sterility barrier between *M. filicifolius* and both *M. guttatus* and *M. laciniatus*. Hybrid sterility is further evidence that *M. filicifolius* is a genetically divergent *Mimulus* species and provides a strong reproductive isolating barrier from near-by *Mimulus* taxa.

2.5 Discussion

2.5.1 *M. filicifolius* and *M. laciniatus* have similar ecology, mating system and genome size

Ecological divergence between closely related species contributes directly to reproductive isolation and may be particularly important during local speciation, as it is in sympatric speciation, given the close geographic location of a newly budded species to its progenitor. This divergence also allows for the coexistence of allopatric species when they come back into secondary contact during range expansion so that they will not have to compete with each other for resources (Barraclough and Vogler 2000, Weir and Price 2011). To assess the amount of ecological divergence among four taxa in the

Table 8. Genetic PCA summary statistics. For both the concatenated *Mg* loci (CONCAT) and CYCA datasets the mean, standard deviation, and 95% Confidence Interval (95% CI) was calculated for PC1, PC2, and PC3 in each species.

		Principal Component 1			Principal Component 2			Principal Component 3		
Locus	Species	Mean	SD	95% CI	Mean	SD	95% CI	Mean	SD	95% CI
CONCAT	*M. guttatus*	-1.84	2.86	[-2.84, -1.21]	0.08	2.98	[-0.59, 0.74]	0.2	2.97	[-0.46, 0.86]
	M. nasutus	5.1	2.6	[3.71, 6.48]	1.92	1.22	[1.27, 2.57]	0.53	0.64	[0.19, 0.87]
	M. laciniatus	8.05	1.22	[7.03, 9.07]	-2.34	1.74	[-3.79, -0.88]	-0.66	1.67	[-2.06, 0.74]
	M. filicifolius	1.76	-	-	-18.35	-	-	-19.42	-	-
CYCA	*M. guttatus*	4.72	1.84	[4.06, 5.37]	-0.06	2.57	[-0.97, 0.84]	-0.79	3.59	[-2.06, 0.48]
	M. nasutus	-5.4	0.6	[-5.65, -5.16]	0.76	0.3	[0.64, 0.88]	0.42	0.37	[0.27, 0.57]
	M. laciniatus	-5.67	0.12	[-5.81, -5.52]	0.49	0.28	[0.14, 0.83]	-0.14	0.52	[-0.79, 0.51]
	M. filicifolius	0.42	-	-	-22.67	-	-	13.58	-	-

M. guttatus species complex whose ranges largely overlap, *M. guttatus, M. nasutus, M. laciniatus,* and *M. filicifolius,* we measured several important ecological variables on a fine environmental scale across populations in the Sierra Nevada Mountains of California. At many of these locations *M. guttatus, M. nasutus,* and *M. laciniatus* occurred sympatrically, but not panmictically. We found that *M. guttatus* and *M. nasutus,* whose ranges are highly overlapping, occupy similar habitats that are cool and wet. *M. laciniatus* and *M. filicifolius*, whose ranges are geographically isolated from one another, both occur in relatively hot, dry, and light intensive habitats (Figure 5). Thus we find that the most genetically distant and allopatric species pair in our analysis, *M. filicifolius* and *M. laciniatus,* is ecologically similar.

In order to further characterize the new morphological species *M. filicifolius* and assess potential sources of reproductive isolation we compared its mating system and genome size to that of *M. laciniatus.* Both species have highly self-fertilizing mating

systems and similar genome sizes. The repeated evolution across taxa of a self-fertilizing mating system from a primarily outcrossing one may allow plants to speciate frequently in small marginal populations. Self-fertilization allows plants to colonize a new habitat with a very small number of individuals, theoretically just one (Baker 1955). An observed pattern in plant species distributions is that self-fertilizing taxa occur in ecologically or geographically marginal habitat compared to their outcrossing relatives (Stebbins 1957). The evolution of self-fertilization is also an effective reproductive isolating barrier from proximate outcrossing species (Kiang and Hamrick 1978, Levin 2010) because gene flow is greatly reduced between self-fertilizing populations and species (Charlesworth and Wright 2001, Sweigart and Willis 2003, Fishman and Wyatt 1999, Martin and Willis 2007). Both *M. laciniatus* and *M. filicifolius* possess small flowers and are highly self-fertilizing with very similar levels of inbreeding. *M. filicifolius'* ability to self-fertilize may have played an important role in its successful colonization of a marginal granite outcrop habitat. The evolution of a highly self-fertilizing mating system would have provided pre-zygotic isolation from nearby outcrossing *Mimulus* and may have contributed to the subsequent genetic divergence of *M. filicifolius* from its progenitor species.

2.5.2 Patterns of Genetic Variation in the *M. guttatus* Species Complex

The *M. guttatus* species complex is a closely related group of wildflowers consisting of the wide ranging and outcrossing *M. guttatus* and many smaller ranged morphological species that are often self-fertilizing. Due to the recent nature of speciation and ongoing interspecific introgression in this group the phylogeny of the species complex remains largely unresolved. However, because of its wide geographic range and high levels of intraspecific genetic diversity it is likely that *M. guttatus* is the progenitor of the other self-fertilizing species with restricted ranges. This makes the *M. guttatus* species complex excellent for both the study of the genetics of recent speciation and local geographic speciation. *M. filicifolius* has recently been described as a new species of *Mimulus* based on divergence in morphological characters from a member of the *M. guttatus* species complex, *M. laciniatus* (Sexton et al 2013). Previously populations of this species were identified as *M. laciniatus* because of similar leaf shape and granite outcrop habitat. This new species occurs only in a few locations in Butte and Plumas counties, CA making it a good candidate for speciation on a local geographic scale. However it was not previously known whether this species was genetically as well as phenotypically distinct from *M. laciniatus* and thus whether it was really a different species. We found strong evidence in our principal components analyses that this species is highly genetically divergent from *M. laciniatus*. In both our genetic principal

components analyses *M. filicifolius* was significantly genetically differentiated from the other three *Mimulus* taxa. In addition *M. filicifolius* was even more genetically distant from the average *M. laciniatus* individual than it was from either *M. guttatus* or *M. nasutus*. These patterns of genetic variation indicate that *M. filicifolius* is indeed genetically distinct from *M. laciniatus,* but it is not possible to determine its closest relative from our current data. The narrow geographic range of *M. filicifolius* compared to other members of the *M. guttatus* species complex is consistent with, although not proof of, it being a product of local geographic speciation.

Given our data there are two main evolutionary scenarios that could have given rise to *M. filicifolius.* The first is that this new species originated from *M. laciniatus* and then subsequently diverged due to geographic isolation over a long period of time. This geographic isolation could have either originated from a long distance dispersal event from *M. laciniatus's* current range or a contraction of a larger historical range. The alternative scenario is that *M. filicifolius* arose as a completely independent lobed-leaved granite outcrop specialist from some other species like the wide-ranging *M. guttatus.* This latter hypothesis is exciting since it would indicate that there has been parallel evolution of lobed leaf shape and granite outcrop specialization in *Mimulus.* The correlation between the independent evolution of a trait and occupation of a similar environment is considered evidence of adaptation (Futuyma 1997). Lobed leaves are in

fact hypothesized to be adaptive in hot, dry environments like *M. laciniatus* and *M. filicifolius'* granite outcrop habitat (Givnish 1987, Nobel 2004).

2.5.3 Hybrid Sterility and Potential Chromosomal Divergence

In our above analyses we found that *M. filicifolius* is genetically as well as phenotypically divergent from *M. laciniatus* and that it has a highly self-fertilizing mating system. Self-fertilizing acts as a pre-zygotic reproductive isolating barrier against nearby outcrossing and self-fertilizing species alike. However for a species to be truly reproductively isolated from its relatives there must be more than one type of isolating barrier. Our discovery of a strong post-zygotic reproductive isolating barrier between *M. filicifolius* and *M. laciniatus* indicates that *M. filicifolius* is highly reproductively isolated and is therefore truly an independent biological species. F1 hybrids in both crosses with *M. filicifolius* produced no autonomously self-fertilized seed and next to none in hand pollinations.

Hybrid sterility has two major causes: chromosomal rearrangements and Dobzhansky-Muller incompatibilities (Coyne and Orr 2004). We do not currently have data that can conclusively tell us whether Dobzhansky-Muller incompatibilities or chromosomal rearrangements are to blame for *M. filicifolius'* hybrid sterility. We did find a small difference in genome size between *M. filicifolius* and *M. laciniatus* in our flow cytometry analysis. However the reduction in *M. filicifolius'* genome size was not far

outside the variation in genome size observed within a single *Mimulus* species (Modliszewski and Willis 2012) indicating that it is unlikely there are chromosomal differences due to large deletions or anueploidy. What is clear is that substantial genetic divergence must have taken place between *M. filicifolius* and *M. laciniatus* for a post-zygotic reproductive isolating barrier of this strength to have arisen.

This study has explored the genetic similarity of and degrees of reproductive isolation between two granite outcrop endemic *Mimulus* taxa with restricted geographic ranges, *M. filicifolius* and *M. laciniatus*. We find that while these two species have highly similar habitats, morphologies, and mating systems they are genetically distinct and reproductively isolated by both pre- and post-zygotic barriers. *M. filicifolius* can therefore be considered the newest biological species in the genus *Mimulus.*

3. THE GENETICS OF LEAF SHAPE DIVERSIFICATION IN THE *MIMULUS GUTTATUS* SPECIES COMPLEX

3.1 Summary

Leaf shape varies extensively across the plant kingdom. There are many hypothesis about the adaptive significance of leaf shape and similar leaf shapes have repeatedly evolved across genera and species. Due to these factors botanists have long been interested in understanding the genetic basis of leaf shape and have made many advances in recent years in model species. However, considerably less is known about the genetic architecture of leaf shape variation in natural populations. In this study we examine the genetic architecture of leaf shape diversification in the *M. guttatus* species complex by genetically mapping quantitative trait loci (QTL's) involved in leaf shape divergence in three species: *M. laciniatus*, *M. nudatus,* and *M. guttatus. M. laciniatus* has a lobed leaf shape and occupies granite outcrops, while *M. nudatus* has a thin elongated leaf and is endemic to serpentine soils. The recently discovered M2L population of *M. guttatus* contains a lobed leaf shape polymorphism. We find that the genetic architectures of these three species' leaf shapes are simple and highly overlapping at the QTL level. We also find several promising leaf shape candidate genes under the largest effect QTL on linkage group 4.

3.2 Introduction

An organism's form is intimately related to its physiological and biomechanical function. Consequently, morphological variation has fascinated evolutionary biologists since Darwin. Leaf shape varies extensively across the angiosperms. There is a tremendous diversity in leaf form within genera and leaf shape polymorphisms are often found within species and populations (Jones et al 2009, Wyatt and Antonovics 1981, Bright and Rausher 2008, B.K. Blackman unpublished data). There is also a great deal of convergence on similar leaf shapes across genera and species. Two main questions have arisen from leaf shape's immense diversification: is this variation adaptive and what is its genetic basis? Two main evolutionary processes give rise to phenotypic variation, natural selection and random genetic drift. Leaves are the major photosynthetic organs of a plant and thus their shape should affect an array of important physiological processes and consequently plant fitness. The adaptive potential of leaf shape and its extensive variation have long interested botanists and evolutionary biologists alike (Vogel 1968, Parkhurst et al 1968, Givnish 1987, Nicotra et al 2011). The repeated independent evolution of leaf shape across taxa provides an excellent opportunity to study the genetics of parallel evolution. Whether convergent phenotypes have the same genetic underpinnings can inform us about the predictability of evolution and the extent of genetic constraint due to antagonistic pleiotropy

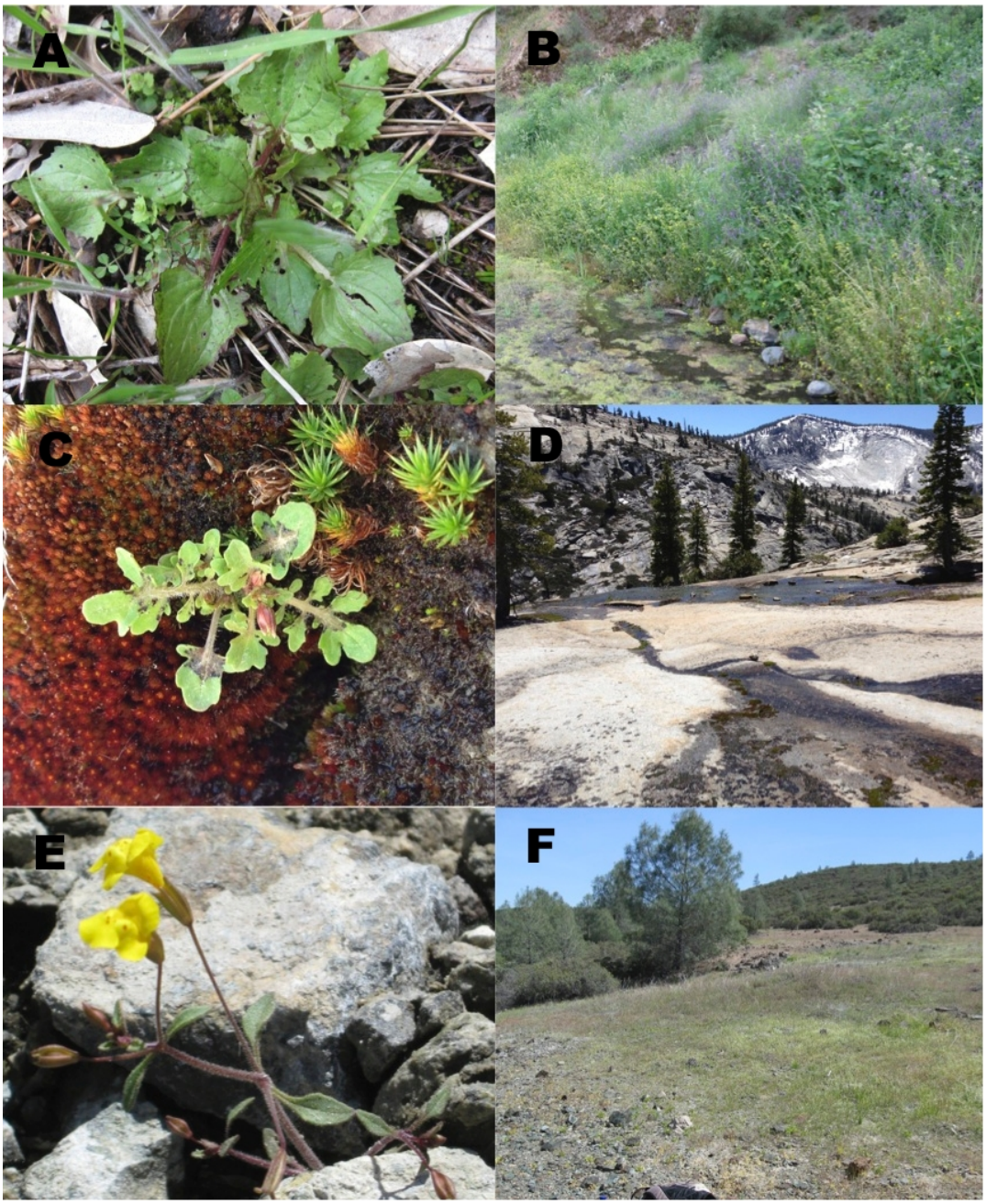

Figure 8. Photographs of A) an *M. guttatus* rosette, B) typical *M. guttatus* seep habitat, C) *M. laciniatus* rosette, D) typical *M. laciniatus* granite outcrop habitat, E) *M. nudatus*, F) typical *M. nudatus* serpentine outcrop habitat.

(Williams 1957, Cooley and Willis 2008).

In the last several decades there has been tremendous interest in the molecular genetic mechanism of leaf shape diversity and great strides have been made towards understanding it. Variation in leaf shape has been genetically characterized in several species, namely *Arabidopsis thaliana*, *Cardamine hirsuta*, and tomato. These studies have found that changes in the leaf margin such as serration, lobing, or leaflet production are due to similar genetic mechanisms across several species indicating that there is some predictability about leaf shape evolution at the molecular level (Bharathan et al 1999, Koenig and Sinha 2010, Scarpella et al 2010, Nicotra et al 2011). Most of these studies have used reverse genetics to find loci that affect leaf shape in crop and model species. Considerably less is known about the genetic architecture of leaf shape variation in natural populations (but see Kimura et al 2008). Are leaf shape differences in nature due to many loci of small effect, or a few of large effect? Do similar leaf shapes have similar genetic architectures across species?

The *Mimulus guttatus* species complex is an excellent system for studying the genetics and evolution of leaf shape diversity. The species complex is a closely related group of highly inter-fertile wildflowers that vary in many traits including leaf shape and ecology. There is also a wealth of genetic tools including the fully sequenced and annotated genome of the IM62 line of *M. guttatus* (Wu et al 2008). The most striking differences in leaf shape in the complex occur between *M. guttatus, Mimulus nasutus, Mimulus nudatus, Mimulus laciniatus,* and the newly described *Mimulus filicifolius. M.*

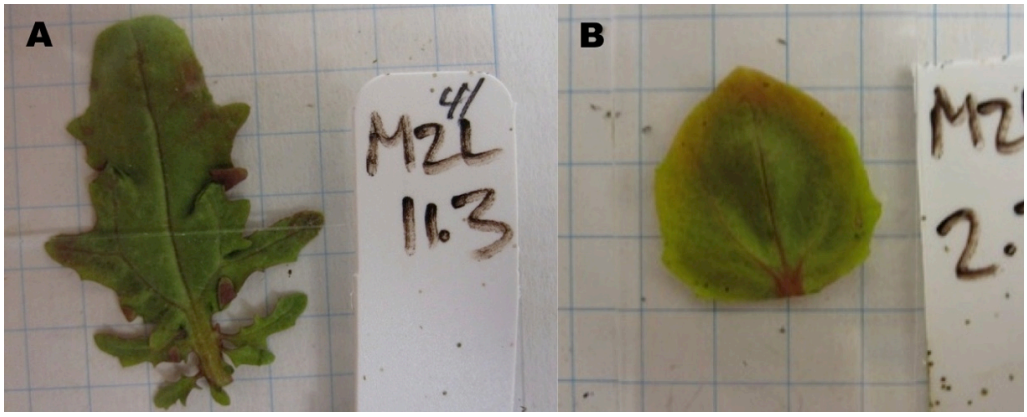

Figure 9. Photographs of leaves from A) a typical lobed-leaved plant in the M2L *M. guttatus* populations and B) a typical round-leaved plant in the M2L population.

guttatus and *M. nasutus* occur in perennially moist streams and seeps and have rounded entire leaves (Figures 8A&B). *M. nudatus* grows in dry, rocky serpentine outcrops and has very narrow leaves (Figure 8E&F). *M. laciniatus* and *M. filicifolius* occur in dry granite outcrops and possess highly lobed leaves (Figure 8C&D). *M. guttatus* is believed to be the progenitor of other species in the complex because of its wide geographic range and high levels of genetic diversity (Sweigart and Willis 2003, Modliszewski and Willis 2012). Regardless of the exact evolutionary history of the species complex, it appears that rounded entire leaves are the ancestral state whereas lobed and narrow leaf margins are derived. Since *M. laciniatus, M. filicifolius,* and *M. nudatus* are edaphic endemics it seems likely that their divergent leaf shapes may be adaptive in their dry, rocky habitats. Narrow and lobed leaves both have thinner boundary layers than rounded entire leaves like those of *M. guttatus*. A thinner boundary layer allows leaves to be cooled more effectively by convection (Givnish 1978, Schuepp 1999, Nobel 2005) and thus may be

adaptive in hot, dry environments. There is a correlation in the *M. guttatus* species complex between reduced leaf boundary layer and occupation of hot, dry rocky habitats.

In addition to the independent evolution of lobed and narrow leaf shapes in different species of the complex we have recently found a single population of *M. guttatus* in western California that is polymorphic for lobed leaf shape (Figure 9). The discovery of the M2L population was exciting because, to the best of our knowledge, it is the first observation of an *M. guttatus* population with lobed leaves. The repeated independent evolution of modified leaf shape in a closely related group of species allows us to study the genetic architecture of leaf shape evolution and to ask whether similar genetic changes underlie leaf shape modifications in closely related species.

To investigate the genetics of leaf shape evolution in the *M. guttatus* species complex we used a quantitative trait locus (QTL) mapping approach. We created mapping populations by crossing *M. guttatus* to *M. laciniatus, M. guttatus* to *M. nudatus,* and a lobed leaf individual from the M2L population to the IM62 inbred line of *M. guttatus.* We attempted to create a mapping population with *M. filicifolius*, but the F1 hybrids in our crosses were sterile so no mapping population could be created (see Chapter 2). We used bulk segregant analysis combined with next generation sequencing (Magwene et al 2011) to quickly map major QTL's for lobed leaf shape in our *M. laciniatus* cross. To look for parallel genetic evolution in our *M. nudatus* and M2L mapping populations we used single pcr-based markers located underneath our *M.*

laciniatus QTL's. If the genetic architecture of leaf shape is similar between species then it may indicate that there are genetic constraints on leaf shape evolution in this group due to antagonistic pleiotropy. Or parallelism at the genetic level may indicate that there is segregating genetic variation for leaf shape within *M. guttatus* and that this variation is repeatedly being captured and selected upon during speciation (Colosimo et al 2005). Without the causal mutations in each species it is not possible to distinguish between these two scenarios, but we are brought one step closer to understanding.

3.3 Materials and Methods

3.3.1 Crossing design and phenotypic analysis

In order to investigate whether the leaf shape diversity among these closely related species was generated through similar genetic pathways we created QTL mapping populations using inbred lines of *M. guttatus*, *M. nudatus,* and *M. laciniatus.* All seeds were cold stratified for at least a week at 4C. *M. laciniatus* parents and hybrids were stratified for 10 days. Plants were grown in the Duke Biology Greenhouses in Fafard 4P potting mix in 2″ pots under 16 hour days. An F2 mapping population of 108 individuals was created for narrow leaf shape by crossing the inbred line DHRO of *M. nudatus* x MED of *M. guttatus* and self-fertilizing F1's. The *M. laciniatus* inbred line WLF47 was crossed to the *M. guttatus* inbred line IM62 to generate F1's which were then self-fertilized to produce ~650 F2's. A lobed leaf line from the M2L population was

crossed to IM62 to produce F1's that were self-fertilized to produce an F2 mapping population of 416 individuals.

Each of these F2 mapping populations was grown up alongside parents and F1's in the greenhouse under 16 hour days and phenotyped for leaf shape. The first or second true leaf was collected from each plant in a grow-out, taped to a piece of white paper, and digitally scanned. The first and second true leaves do not systematically differ in their leaf shape. Narrow leaf shape in the *M. nudatus* x *M. guttatus* cross was quantified by digitally measuring the length and width of each leaf in the program ImageJ v1.47 and computing the length to width ratio for the parental and F2 generations. Lobed leaf shape was quantified for both *M. laciniatus* and M2L crosses in ImageJ using a convex-hull analysis. In this analysis ImageJ creates a convex-hull shape from the leaf image by connecting the outermost points of the leaf. To determine the degree of leaf lobing the area of the actual leaf was subtracted from the area of the leaf's convex-hull and then divided by the area of the convex-hull to control for size. This measurement was then log transformed to normalize the variance in the parental generations. Histograms of leaf shape distributions were created in R.

3.3.2 Quantitative trait locus mapping using bulk segregant analysis and next generation sequencing

To map lobed leaf shape in the *M. laciniatus x M. guttatus* F2 population we used a combination of bulk segregant analysis and single nucleotide polymorphism (SNP) markers created via Illumina sequencing (Magwene et al 2011, Friedman and Willis

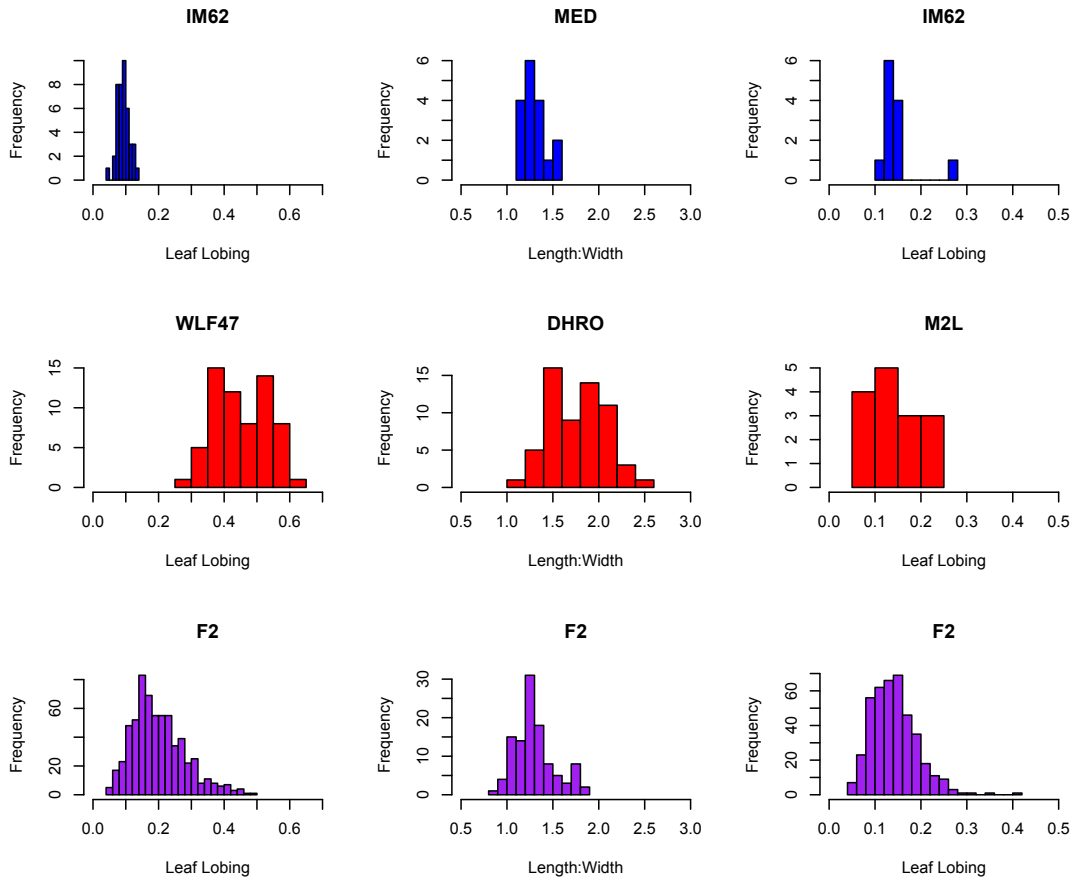

Figure 10. Leaf shape distributions for the three mapping crosses. Column A: Leaf shape was measured by using convex hull analysis on the IM62 *M. guttatus* inbred line (blue), the WLF47 *M. laciniatus* inbred line (red), and the IM62xWLF47 F2 population (purple). Column B: Leaf shape was measured by taking the length to width ratio on one leaf of each plant of the MED *M. guttatus* inbred line (blue), the DHRO *M. nudatus* inbred line (red), and the MEDxDHRO F2 population (purple). Column C: Leaf shape was measured again with convex hull analysis on the IM62 round-leaved inbred line (blue), the M2L lobe-leaved inbred line (red), and the round x lobed-leaved M2L F2 population (purple).

2013). We created two pools of 100 F2's, a highly lobed pool and un-lobed pool. Equal amounts of leaf and bud tissue were collected from each individual. DNA was extracted from each F2 using a modified CTAB protocol (Kelly and Willis 1998). DNA from each

individual was then combined to form two pooled DNA samples, one lobed and one unlobed. Each pooled sample was sequenced in one lane of an Illumina GAII machine using single end reads at the Duke University Sequencing and Analysis Core Resource.

Bulk segregant analysis (BSA) has been used for a long time to roughly map quantitative trait loci (QTL's) of moderate to large effect (Michelmore et al 1991). With the advent of next generation sequencing it is possible to quickly and cost effectively generate dense single nucleotide polymorphism (SNP) markers across the entire genome. Using dense SNP markers in BSA makes it possible to quickly map QTL's at a finer scale (Magwene et al 2011). To map QTL's involved in lobed leaf shape in *M. laciniatus* we first aligned short sequence read files from both pooled samples to the IM62 *M. guttatus* reference genome (www.phytozome.net) using BWA (Li & Durban 2010). We then used SAMtools (Li et al. 2009) to create a pileup file of the combined read files. We ignored sites with <4X coverage and called SNPs as either "IM62" (*M. guttatus)* or "other" based on the reference genome sequence. We calculated IM62 SNP allele frequencies across the genome using the allele counts at each SNP in the pileup file in a sliding window analysis with a window size of 1000 SNPs (B.K. Blackman and L. Flagel, unpublished data). We used a sliding window to calculate allele frequency on account of the low sequence coverage at each individual SNP. We then calculated the difference in IM62 allele frequency between the un-lobed and lobed pools across the genome. At neutral unlinked SNP markers there should be essentially no difference in allele

frequency between leaf shape pools, however markers that are closely linked to QTL's should differ noticeably in allele frequency. We chose an allele frequency difference of 0.25 as the cutoff for selecting markers potentially linked to QTL's. These QTL's were further verified by additional PCR-based marker analysis (see below).

3.3.3 Single marker QTL analysis

Bulk segregant analysis does not allow us to make inferences about the phenotypic effects of individual QTL's. To verify our bulk segregant QTL's and determine the effect size and dominance interactions at individual *M. laciniatus* leaf shape QTLs we genotyped 300 random IM62 x WLF47 F2's at genic markers under each QTL. We screened parental inbred lines for polymorphism at exon-primed intron crossing (EPIC) markers derived from expressed sequence tags (ESTs) (Fishman et al 2008). Polymorphism at each marker was determined by variation in PCR fragment length, which is usually due to insertion/deletion (indel) variation in introns. Primers for these markers can be found on the Mimulus Evolution website (http://www.mimulusevolution.org). PCR products underwent capillary electrophoresis and fragment analysis on an ABI 3730xl DNA Analyzer (Applied Boisystems, Foster City, CA, USA). Fragment size was scored in the program GeneMarker (Soft Genetics, State College, PA, USA.). We verified bulk segregant QTLs using one-way analysis of variance (ANOVA) in JMP v9 (SAS, Cary, NC, USA) at each marker with marker

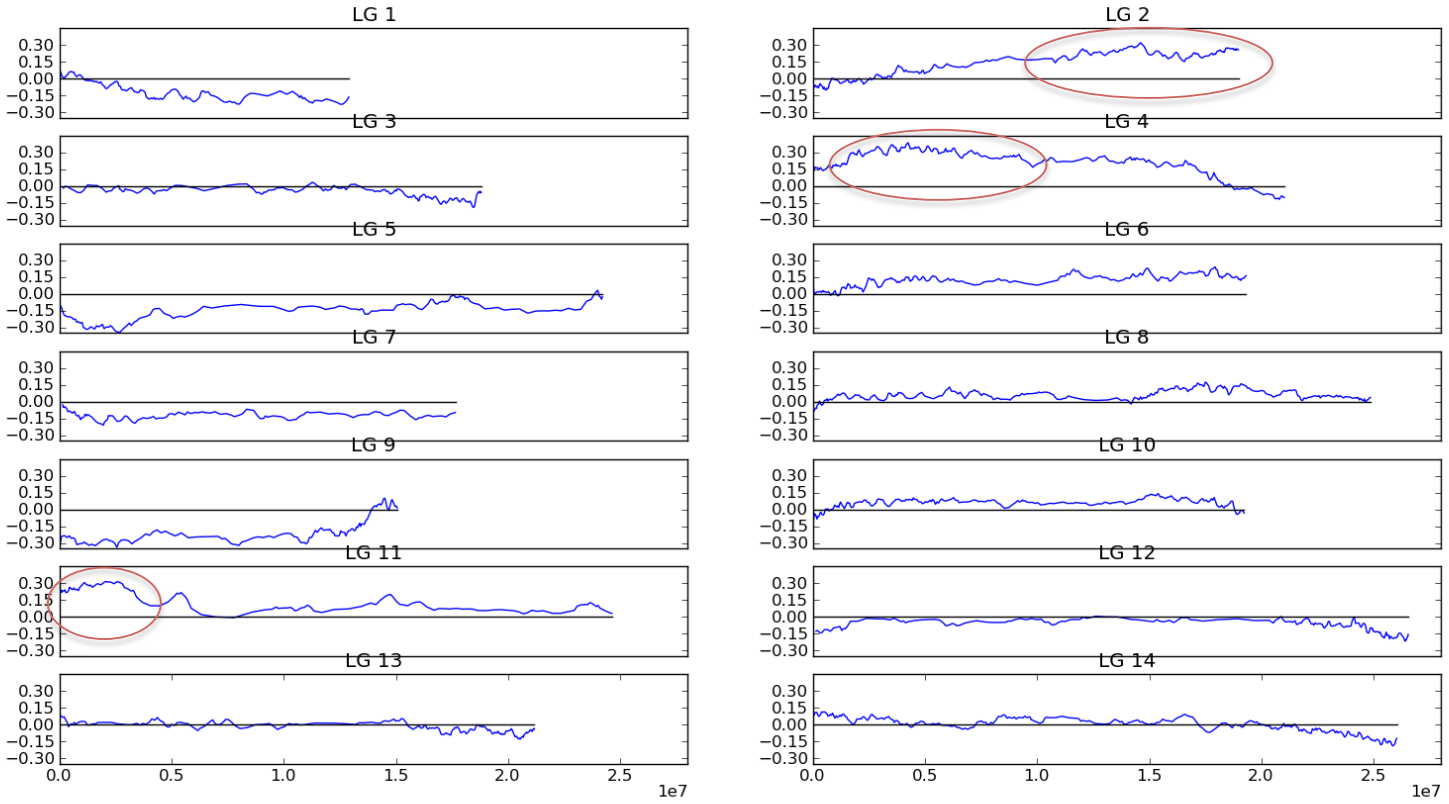

Figure 11. QTL results from the bulk segregant analysis. The blue line is a plot of the different in IM62 allele frequency between the lobed and un-lobed leaf shape pools. To find QTL regions a cut-off of 25% difference in allele frequency was used. Areas surrounded by a red circle are QTL regions that were later confirmed by single marker analysis.

genotype as the independent variable and leaf shape as the dependent. Effect size was estimated at each QTL by calculating the proportion of the segregating variation in leaf shape explained by the most significant marker (R^2). Genotypic values for each QTL were calculated as the mean leaf shape value for each genotype. To test for epistasis between QTL's we looked for significant interaction terms in a multifactor ANOVA in JMP.

To determine whether divergent leaf shapes in *M. nudatus,* the M2L *M. guttatus* population, and *M. laciniatus* have a similar genetic basis we performed single marker analysis in M2L and *M. nudatus* F2 populations at our known *M. laciniatus* leaf shape QTL's. We genotyped 108 *M. nudatus x M. guttatus* and 384 M2L x IM62 F2's at three polymorphic markers in the genomic region beneath each *M. laciniatus* QTL. One-way ANOVA's were performed in JMP to determine whether there was a statistically significant association between each marker and leaf shape phenotype in each cross. Effect size, genotypic value of, and epistasis between each QTL were determined as described above in our *M. laciniatus* single marker analysis.

3.4 Results

3.4.1 Analysis of phenotypic variation in leaf shape

Analyzing the phenotypic variation in parental and F2 hybrid generations allows us to make inferences about the underlying genetic architecture of a trait. In our *M. laciniatus* cross the phenotypic distributions of leaf lobing in our parental and F2 generations indicate several things. One is that *M. laciniatus's* leaf phenotype is more environmentally variable than *M. guttatus'* (Figure 10). We know this because phenotypic variation in an inbred line like WLF47 must be due entirely to environmental variance (VE) since all individuals are genetically identical (Falconer and MacKay 1996). Another pattern that can be identified right away is that the F2 leaf shape distribution overlaps substantially with both parental distributions (Figure 10). This indicates that leaf shape is a genetically simple trait in *M. laciniatus*. The broad sense heritability of lobed leaf shape in our *M. laciniatus* x *M. guttatus* cross is 76.6%. In our *M. nudatus* cross we see similar patterns with *M. nudatus'* leaf shape being more phenotypically variable than *M. guttatus'* and the F2 distribution largely overlapping both parental distributions (Figure 10). The broad sense heritability of narrow leaf shape in our *M. nudatus* x *M. guttatus* cross is 23%**.** The genetics of the M2L cross are difficult to describe from the given phenotypic distribution because the actual parental line from M2L died out and could not be phenotyped in large numbers. The M2L line that was

grown with the F2 grow-out is not very lobed. There could be a problem with our leaf shape metric in this cross because lobing is subtler in M2L than in *M. laciniatus* and the un-lobed parent, IM62, is highly serrated. This seems to be causing noise in our measurements because one un-lobed IM62 parent appears more highly lobed than any of the supposedly lobed M2L parents. However we can still tell that there is a lot of environmental variance in the lobed M2L's leaf phenotype and, except for one outlier, relatively little in the un-lobed IM62's leaf phenotype (Figure 10). The broad sense heritability of lobed leaf shape in our *M2L* x *IM62* cross is 21%.

3.4.2 Leaf shape in *M. laciniatus* is genetically simple

Using a combination of bulk segregant analysis and SNP markers generated through Illumina sequencing we mapped five putative leaf shape QTL's in our *M. laciniatus x M. guttatus* F2 population (Figure 11). We were able to confirm three of these QTL's with single marker analysis in a random selection of F2's (Table 9). The largest effect QTL is located on LG 4 (R^2=11%), the second largest effect on LG2 (R^2=7.7%), and the QTL of smallest effect on LG11 (R^2=2%). QTL's on LG2 and LG4 both cover wide genomic regions. This may be because there were difficulties extracting high quality DNA from individuals in the leaf shape pools. Due to differences in DNA quality a few individuals may be driving the difference in allele frequency between the pools, which would create a lower resolution QTL map. The QTL on LG4 spans most of the chromosome with a

small dip in allele frequency difference near the centromere. It is hard to tell from this analysis if LG4 is one large or two separate QTLs because we lack sufficient recombination resolution. We found no evidence of epistasis between any of the three genomic regions. The lobed *M. laciniatus* allele is dominant at QTL's on LG's 2 & 4. The mean phenotype of the heterozygote at each of those loci is close to the mean phenotype of the *M. laciniatus* homozygote (Table 9). The heterozygotes at the LG11 QTL are intermediate indicating that this locus is co-dominant.

3.4.3 Parallel evolution at the QTL level

We found evidence of parallel leaf shape evolution at the QTL level in both our *M. nudatus* and M2L mapping populations. Markers beneath *M. laciniatus* QTL's on LG2 & 4 were significantly associated with narrow leaf shape in the *M. nudatus x M. guttatus* F2 and markers beneath all three QTL's (LG2, LG4, & LG11) were significantly associated with lobed leaf shape in the M2L F2. *M. nudatus* leaf shape QTL's were of roughly equal effect (LG2=8%, LG4=4%, Table 9). The heterozygotes at each locus were intermediate between the homozygotes so there was no evidence of dominance in the narrow leaf shape phenotype. There was no evidence of epistasis between *M. nudatus* leaf shape QTL's. The R^2 values for the QTL's in the M2L F2 population were of equal and small effect (LG2=2%, LG4=2%, Lg11=2%). There was evidence of over-dominance a

Table 9. Marker and QTL data. For each experimental cross we have listed the marker name, the base pair position and linkage group of the markers location, the R^2 from our logistic regressions of marker genotype on leaf phenotype, the proportion of mean parental difference was calculated by subtracting the mean homozygote phenotypic values and dividing this difference by the difference between the mean parental phenotypic values, F-ratios are from our logistic regressions (*denotes the level of significance), we have also listed the mean phenotype for each genotypic class in each cross at each marker.

Experimental Cross	Marker	Linkage Group	Position (bp)	R^2	Proportion of mean parental diff	F ratio	Mean Phenotype Lobed/Narrow Homozygote	Mean Phenotype Heterozygote	Mean Phenotype Round Phenotype
M. laciniatus x M. guttatus	MgSTS192	2	17592566	0.077	20%	10.37****	-0.694	-0.706	-0.82
	MgSTS262	4	5222698	0.11	22%	10.99****	-0.644	-0.699	-0.798
	MgSTS644	11	778805	0.02	10%	2.6*	-0.68	-0.717	-0.749
M. nudatus x M. guttatus	MgSTS184	2	3093681	0.043	31%	2.09 NS	1.356	1.305	1.21
	MgSTS81	4	903396	0.078	40%	3.44*	1.415	1.296	1.225
	MgSTS184	2	-	0.043	-	3.99*	-	-	-
	MgSTS81	4	-	0.075	-	6.68*	-	-	-
M2L x IM62	MgSTS530	2	18312396	0.019	NA	3.35*	0.134	0.143	0.153
	MgSTS306	4	3482954	0.02	NA	3.32*	0.143	0.149	0.132
	MgSTS26	11	339314	0.019	NA	3.23*	0.15	0.143	0.129

the QTL on LG4 because the heterozygote leaf shape mean was higher than that of the lobed parent (Table 9). The M2L leaf lobing QTL on LG2 was negative, meaning that homozygotes with the un-lobed allele were more lobed than homozygotes with the lobed allele. There was no evidence of epistasis between any of the M2L leaf shape QTL.

3.5 Discussion

We found that leaf shape is a genetically simple trait in the *M. guttatus* species complex. In each of our three mapping populations there was substantial overlap between the phenotypic distributions of the parental and F2 generations. This indicates that leaf shape is genetically simple because the fewer loci involved in a trait the greater the variance in the F2 is compared to the difference between the parental phenotypes (Castle 1921, reviewed in Lynch and Walsh 1998). We also found that leaf lobing and narrowness are subject to substantial environmental variation. Despite increased environmental variance in the divergent leaved parent broad sense heritabilities were high in our *M. laciniatus* cross and moderate in the *M. nudatus* and M2L crosses. Interestingly we found a large degree of parallelism between the genetic architecture of lobed leaves in *M. laciniatus,* narrow leaves in *M. nudatus,* and lobed leaves in the M2L population of *M. guttatus* at the QTL level. We found evidence of dominance of lobed leaf shape at two *M. laciniatus* leaf shape QTL's, no evidence of dominance in *M. nudatus*, and evidence of over-dominance at one QTL in the M2L population. There were

no significant interactions among any of the QTLs indicating that epistasis does not substantially contribute to the expression of these leaf phenotypes.

3.5.1 The genetic basis of lobed leaf shape in *M. laciniatus*

We mapped three major QTL's underlying lobed shape in *M. laciniatus*. These three QTL's explain ~20% of the leaf shape variation in the F2 population. This is a lower number than we had expected given the large degree of overlap between the parental and F2 phenotypic distributions. However there are several reasons why these R^2 values might be low at these markers. One is that our QTL regions are still very large so the markers that we are estimating effect size with may be quite distant from the causal locus or loci. This means there would have been recombination between our markers and the causal mutation(s), which would lower our R^2 significantly (Falconer and Mackay 1996). Another reason is that there could be other modifier QTL's of small effect contributing to this phenotype that could not be detected by bulk segregant analysis. We have no doubt that these loci exist and perhaps even vary between populations of *M. laciniatus,* however it seems unlikely that they alone account for the difference between the total R^2 and the broad sense heritability (Table 9).

We found several promising leaf shape candidate genes under the QTL on LG4. We found an ortholog of the ROTUNDIFOLIA4 (ROT4) gene in *Arabidopsis thaliana, which* is involved in elongation of the leaf through cell proliferation. Loss of function

mutations in or overexpression of ROT4 causes leaves to be short and wide (Tsukaya 2006). In addition to *M. laciniatus's* leaves being lobed they are also more elongated than *M. guttatus's* and our convex hull measurement of lobing also seems to capture differences in leaf elongation. This is because a leaf is judged as being more lobed the greater the difference is between the areas of the normal leaf and the convex hull. That difference is actually maximized the more space there is between leaflets and there is more space between leaflets in longer leaves.

A promising candidate gene for leaf lobing is the tomato KNOX gene PETROSELINUM (PTS). Up-regulation of this gene is responsible for an increase in leaf complexity between two wild species of tomato (Kimura et al 2008). Another candidate gene for leaf lobing under our QTL on LG4 is PIN1. The PIN1 protein causes the formation of auxin maxima in the shoot apical meristem (SAM) and within the developing leaf. These auxin maxima drive the formation of lateral organs from the SAM and vasculature, serrations, lobes, and leaflets in the developing leaf (Koenig and Sinha 2010, Scarpella 2010). There are also four putative TCP transcription factors beneath the QTL on LG4 and these are involved in leaflet initiation in tomato's compound leaves. Finally there is a paralog of the NAC transcription factor GOBLET (GOB) that is involved in compound leaf development in tomato through repression of auxin between initiating leaflets beneath the same QTL on LG4 (reviewed in Koenig and

Sinha 2010). No candidate genes for leaf shape have been found below our BSA QTL's on either LG2 or LG11. It is interesting that orthologs of so many genes involved in leaf shape evolution seem to cluster on LG4.

3.5.2 Evidence of parallel evolution at the QTL level

We found overlap in leaf shape QTL's between *M. laciniatus*, *M. nudatus*, and the *M. guttatus* population M2L. This is evidence of parallel leaf shape evolution at the QTL level. QTL regions on LG2 & 4 were significantly associated with variation in leaf shape in all three of our F2 mapping populations. This overlap was initially unexpected because the narrow leaf shape of *M. nudatus* is phenotypically distinct from the lobed leaf shape of *M. laciniatus* and M2L. However upon closer consideration of our convex hull leaf shape metric it seems that we may be capturing leaf elongation in addition to lobing. And both *M. laciniatus* and the lobed form of M2L have leaves that are more elongated than *M. guttatus*. In order to tease apart these two correlated shape characteristics it would be necessary to use a different metric of leaf lobing, e.g. number of lobes per leaf, and separately measure length to width ratio.

It is also possible that the LG2 or LG4 QTL regions contain two separate loci, one responsible for leaf elongation and one for leaf lobing. This seems likely given the variety of candidate genes involved in various parts of leaf development underneath the QTL on LG4. ROT4 seems like a particularly good candidate gene for leaf elongation in

M. nudatus. A third possibility is that elongation and lobing are in fact controlled by one pleiotropic locus such as a transcription factor regulating the auxin pathway like GOB. Further functional studies such as qPCR, sequencing, and transformation would be necessary to determine if these candidates control leaf shape in these species and whether QTL overlap is due to pleiotropy.

All three of *M. laciniatus* 's leaf shape QTL's were significantly associated with leaf shape variation in the M2L population of *M. guttatus*. This is less surprising than the observed QTL parallelism in *M. nudatus* because the lobed leaf phenotypes are very similar, however it is no less exciting. The M2L population is polymorphic for lobed leaf shape. The fact that M2L shares a genetic mechanism for lobed leaves with *M. laciniatus* suggests that *M. laciniatus's* leaf shape could have been derived from segregating variation in *M. guttatus.* This may explain why lobed leaf shape has evolved multiple times in the *M. guttatus* species complex in *M. laciniatus* and *M. filicifolius* (Ferris et al in prep).

It has been demonstrated in other species that an adaptive trait evolved multiple times independently by drawing on segregating variation in the ancestral population. In marine sticklebacks there is segregating variation for lateral plate size at the EDA locus in the large marine population. In the marine form lateral plates are large. The freshwater form has evolved reduced lateral plate size multiple times during the

independent colonization of rivers through selection on segregating variation at the EDA locus (Colosimo e al 2005). Alternatively M2L and *M. laciniatus* may have evolved lobed leaves independently, but through similar genetic mechanisms. This could be evidence of evolutionary constraint via negative pleiotropy. We cannot distinguish between these two evolutionary scenarios in the *M. guttatus* species complex because 1) we do not understand the genetic basis of *M. filicifolius's* leaves to due a post-zygotic isolation between it & other members of the species complex (see Chapter 2), and 2) we lack the causal loci and mutations underlying the different leaf shapes.

Leaf shape diversity is extensive across and within angiosperm species. This diversification is fascinating because of leaf shape's affect on physiology and potential affect on fitness. Similar leaf shapes have evolved many times independently between species of the same genus. Investigating the genetic basis of leaf shape allows us to understand the degree of genetic parallelism in the evolution of a potentially adaptive trait. We found that evolution of both divergent and convergent leaf shapes is due to similar genetic mechanisms at the QTL level in the *M. guttatus* species complex. These QTL could each harbor several causal loci indicating that loci involved in leaf development in different species cluster together in the same genetic regions. Or QTL overlap between species may mean that the same genes are being modified in each species to produce different leaf shapes. This contributes to our knowledge of the

genetics of leaf shape diversification and parallel evolution at the level of genetic architecture.

4. The genetics of adaptation to granite outcrop environments

4.1 Summary

A primary goal in evolutionary biology has been to understand what traits and genes underlie local adaptation to different environments. This is difficult since many traits diverge simultaneously between populations and species. Using genetic manipulation in combination with measurements of fitness in the field allows us to investigate a traits individual adaptive significance. In this study we investigate which traits and genetic regions underlie adaptation to a dry, exposed granite outcrop environment in *Mimulus laciniatus.* We use next generation sequencing to map quantitative trait loci (QTL's) involved in flowering time, flower size, and leaf shape divergence between *M. laciniatus* and a sympatric population of its close relative *M. guttatus* in the greenhouse, measure natural selection on flowering time, flower size, and leaf shape in a reciprocal transplant using *M. laciniatus x M. guttatus* F4 hybrids, and test whether greenhouse QTL's are involved in F4 fitness in the field. We find that divergence in flowering time, flower size, and leaf shape between these two species is due to relatively few and large effect QTL's including a large effect highly pleiotropic QTL on chromosome 8. From our reciprocal transplant we learned that *M. laciniatus* has significantly higher fitness in granite outcrop habitats than *M. guttatus* and that earlier flowering time is adaptive in *M. laciniatus's* habitat. From our genotypic analysis in the

field we learned that genotype at our pleiotropic greenhouse QTL does not contribute to differential habitat adaptation between *M. laciniatus* and *M. guttatus* through antagonistic pleiotropy.

4.2 Introduction

Closely related populations or species often occupy ecologically disparate habitats. How do different populations or species adapt to these new and initially stressful environments? What genes evolve in response to this novel selection pressure and what traits do they influence? These are exciting questions since adaptation to a new habitat can maintain genetic variation within species (Cain and Sheppard 1953, Levene 1953, Rausher 1983, Gillespie and Turelli 1988, Hedrick 2006) or eventually lead to ecological speciation (Mayr 1947, Mayr 1949, Schluter 2001, Coyne and Orr 2004, Rundle and Nosil 2005). Adaptation to different habitats at the organismal level can be accomplished through fitness trade-offs at particular loci (antagonistic pleiotropy) or conditional neutrality where an allele has a fitness advantage in its native environment but is neutral in the other (Figure 12). Antagonistic pleiotropy can maintain genetic variation within a species, conditional neutrality will not. To test which of these factors is responsible for differential habitat adaptation it is necessary to test the fitness affects of alleles in their natural environments (Anderson et al 2011). Many studies have demonstrated that different species or populations are adapted to a particular habitat

through reciprocal transplant experiments, but few have elucidated the specific genes and traits that contribute to this adaptation (reviewed in Kawecki and Ebert 2004 & Hereford 2009). Conversely, the genetic basis of many potentially adaptive traits have been uncovered in the lab, but the fitness effects of these loci are rarely tested in nature (Johanson et al 2000, Nachman et al 2003, Colosimo et al 2005, Rosenblum et al 2010, Chan et al 2010, but see Bright and Rausher 2008). Combining QTL mapping, genetic manipulation, and field experiments can disentangle loci from their genetic backgrounds, elucidate the individual loci and traits that contribute to adaptation, and test whether differential habitat adaptation is produced by antagonistic pleiotropy or conditional neutrality.

Plant species are excellent systems for studying the genetics of differential habitat adaptation. Because of their sessile lifestyle, plants often experience strong

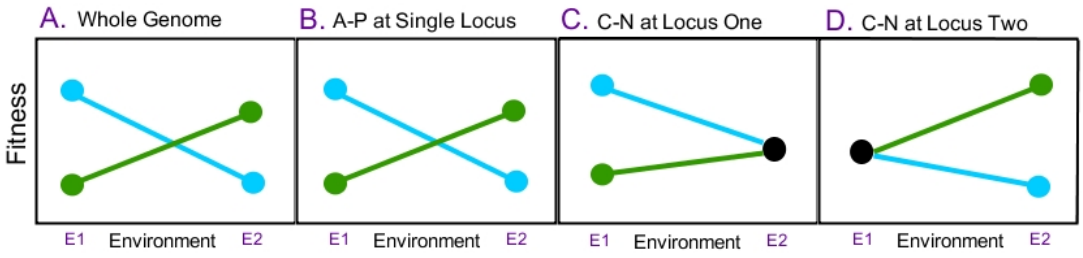

Figure 12. Reaction norms of different genotypes (A) or alleles (B,C, and D) in two environments at A) the whole genome level, B) a single locus demonstrating antagonistic pleiotropy, C) locus one illustrating conditional neutrality, and D) locus two illustrating conditional neutrality.

divergent selection across heterogeneous environments on a small geographical scale (Kalisz 1986, Schmitt and Antonovics 1986, Stewart and Schoen 1987). Members of the *Mimulus guttatus* species complex occupy a variety of edaphic environments. Most members of the species complex occur in moist seep and streambeds, but some occur in rapidly drying habitats such as serpentine soils, copper mine tailings, and granite outcrops. While adaptation to serpentine and copper has been well studied in *Mimulus* little is known about adaptation to granite outcrops. Granite outcrops are characterized by shallow rocky soils, high light intensity, and low soil water retention. The Sierra Nevada native *Mimulus laciniatus* is the most famous granite outcrop endemic in the *M. guttatus* species complex, but we have recently discovered an independent taxon, *Mimulus filicifolius*, that also occurs in a granite outcrop habitat. *M. laciniatus* and *M. filicifolius* possess traits that are likely adaptive in granite outcrops where soils are only a few millimeters deep and dry rapidly once the seasonal snowmelt is gone. These traits are rapid flowering under shorter critical photoperiods (Friedman and Willis 2013) and a self-fertilizing mating system. Both taxa also have a lobed leaf shape that distinguishes them from the rest of the genus *Mimulus* and could be an important adaptive trait in these habitats for reasons listed below. The closely related round-leaved *M. guttatus* occurs in deeper, moist seep and streambed soils within meters of these granite outcrops (Figure 13).

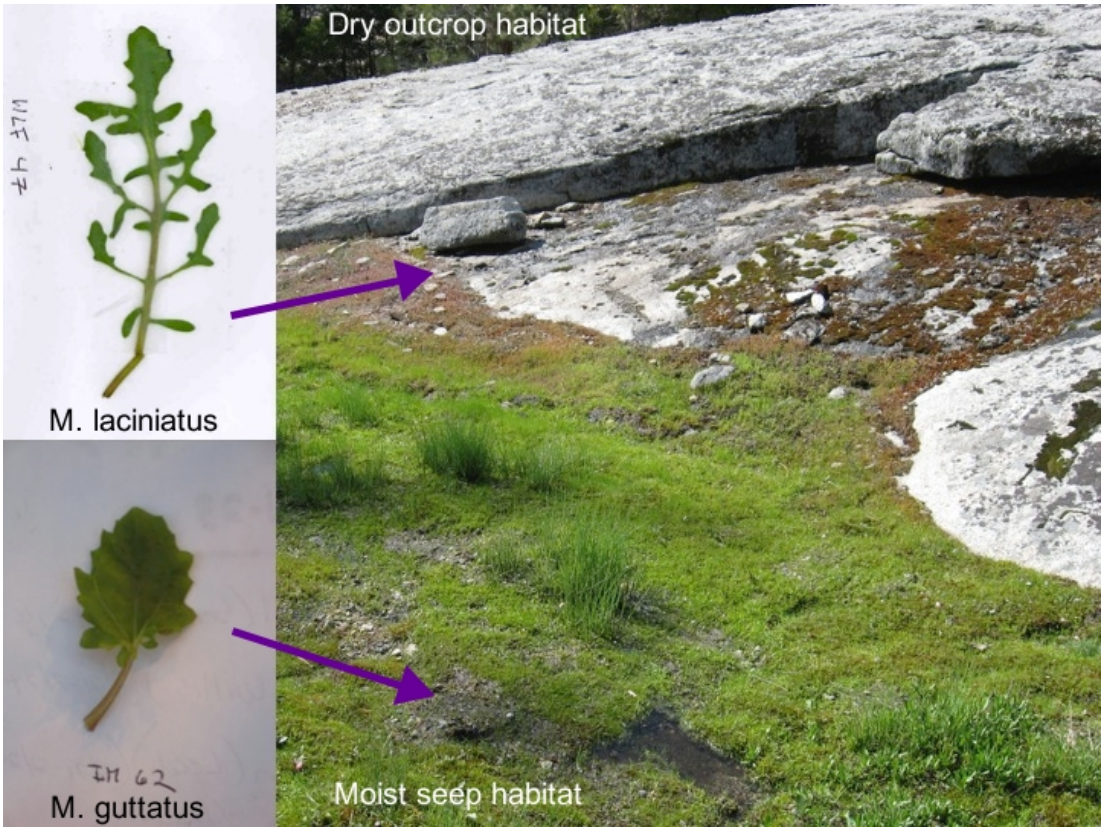

Figure 13. *M. guttatus* and *M. laciniatus* leaf shapes and habitats.

This study addresses how plants adapt to granite outcrop environments by dissecting the genetic bases of flowering time, floral traits associated with self-fertilization, and leaf shape, and testing the adaptive significance of alleles underlying these traits in the field. Traits that are likely to be adaptive in these habitats include early flowering, self-fertilization, and leaf shape. Early flowering allows plants to reproduce before the onset of summer drought (Kiang and Hamrick 1987, Fox 1990, Macnair and Gardner 1998, Hall and Willis 2006). Self-fertilization is often associated

with occupation of drier habitats (Kiang and Hamrick 1987, MacNair and Gardner 1998, Mazer et al 2010, Wu et al 2010). Small flower size and rapid floral development are correlated with self-fertilization and may reduce floral tissue transpiration costs (Galen 1999). Leaf shape varies extensively within and between plant species. Because leaf shape affects the physical properties of a leaf, and leaves are the primary site of gas exchange, water loss and photosynthesis, it should affect plant fitness. Lobed leaves are a striking example of leaf shape variation and have been of particular interest to plant evolutionary biologists (Ashby 1948, Vogel 1968, Givnish and Vermeij 1976, Robichaux 1990, Niklas 1989, Baker-Brosh and Peet 1997).

Since lobed leaf shape is unique to granite outcrop *Mimulus* taxa and is seemingly an example of parallel phenotypic evolution it is likely a key trait in adaptation to this habitat. Leaf shape affects the thickness of the leaf boundary layer with lobed leaves having thinner boundary layers than round leaves (Givnish 1978, Schuepp 1993, Nobel 2005). A thin boundary layer allows a leaf to be heated or cooled more efficiently by convection (Givnish 1978, Nobel 2005). This can prevent water loss through transpirational cooling or keep leaves above freezing when they radiate heat to a cold night sky which could both be advantageous in *M. laciniatus's* exposed, dry granite outcrops.

To understand what traits are adaptive in granite outcrop habitats and whether differential habitat adaptation is due to antagonistic pleiotropy or conditional neutrality at individual loci this study aims to **1)** Determine the genetic basis of ecologically important traits in the greenhouse using next generation sequencing and QTL mapping, **2)** Test for local adaptation and phenotypic selection on putatively adaptive traits in a reciprocal transplant in the field using parental inbred lines and F4 hybrids, and **3)** Test for genetic overlap between trait QTL's in the greenhouse and fitness QTL's in the field.

4.3 Materials and Methods

4.3.1 Study System

The Mimulus guttatus species complex is a group of closely related wild flowers that occurs throughout western North America. This group of species differs dramatically in ecology, morphology, life history, mating system, and possesses a wealth of genetic tools including the fully sequenced and annotated genome of the IM62 inbred line of *M. guttatus*. The combination of so much phenotypic and ecological diversity and an extensive genetic tool kit makes this species complex an ideal system for studying the genetics of adaptation (Wu et al 2007). *M. guttatus* is a widespread outcrossing species that lives in moist seeps and stream beds from Mexico to Alaska and Colorado to the Pacific Ocean. *M. guttatus* has large flowers associated with its highly outcrossing mating system (Willis 1993a) and rounded entire leaves like the majority of

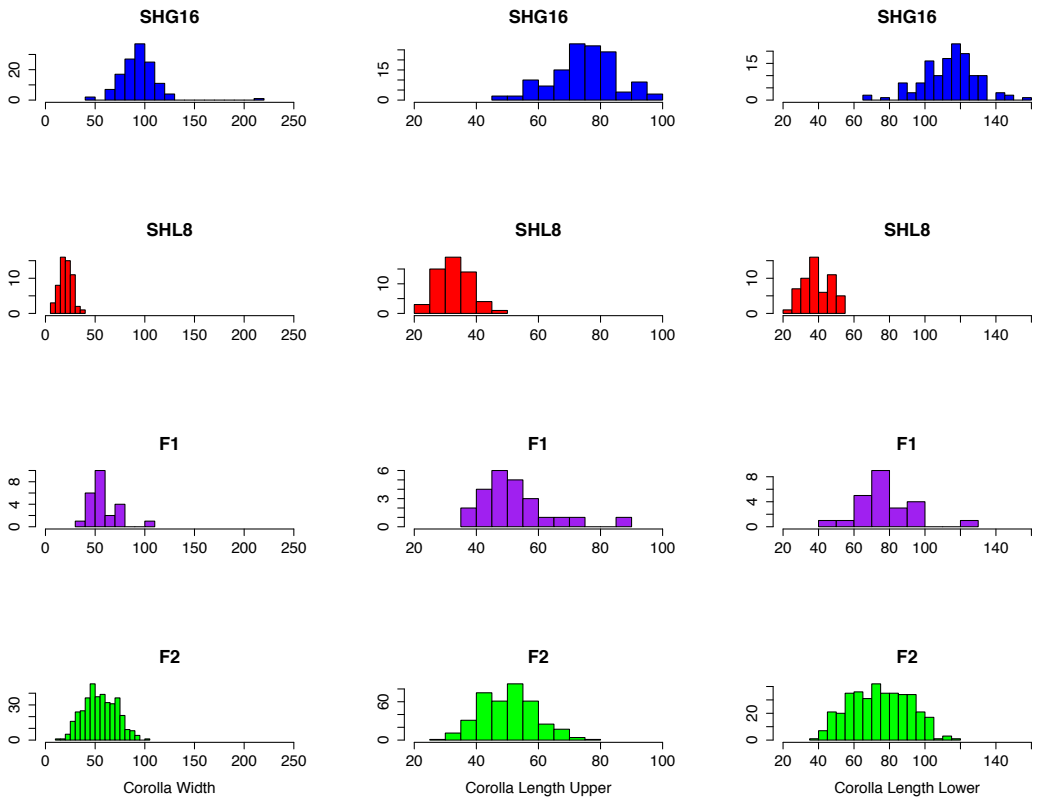

Figure 14. Phenotypic distributions of corolla width, corolla length upper, and corolla length lower for M. guttatus (blue), M. laciniatus (red), F1 (purple), and F2 (green) populations.

the species complex. *Mimulus laciniatus* is a self-fertilizing member of the species complex that occurs in dry, rocky granite outcrops throughout the central and southern Sierra Nevada Mountains, California. *M. laciniatus* has small flowers associated with its highly self-fertilizing mating system and flowers earlier in the season that nearby *M. guttatus* populations. *M. laciniatus* also possesses a distinctive lobed leaf shape. There is only one other species in the genus Mimulus that possesses a lobed leaf shape and that is

a recently described member of the *M. guttatus* species complex, *M. filicifolius*, and it also grows in dry granite outcrop habitat. *M. guttatus* and *M. laciniatus* often co-occur within meters of each other with *M. laciniatus* occupying mossy habitat the middle of the granite outcrop and *M. guttatus* growing alongside the granite in a grassy seep. Inbred lines used in our crosses between these species were derived from two populations of M. guttatus (Yosemite Valley Overlook (YVO), Shaver Lake (SHG)) and two populations of M. laciniatus (White Wolf (WLF), Shaver Lake (SHL)) in close geographic proximity in the central Sierra Nevada Mountains, CA.

4.3.2 Construction of the mapping population

In order to examine the genetic architecture of ecologically relevant differences between *M. guttatus* and *M. laciniatus* in a common garden I created an F2 mapping population between inbred lines from a sympatric *M. guttatus* (SHG) and *M. laciniatus* (SHL) population. In this population *M. laciniatus* and *M. guttatus* individuals occur less than one meter apart. The parental lines SHL8 and SHG16 were obtained from field collected seed/live plants and grown in the Duke University greenhouse for two to three generations before being reciprocally cross pollinated. First generation hybrids (F1) were grown in the greenhouse and self-fertilized to produce a large F2 mapping population.

One thousand F2s were grown up in the Duke University Greenhouse and phenotyped for leaf shape, flower size, node of first flower, and flowering time. F2 and

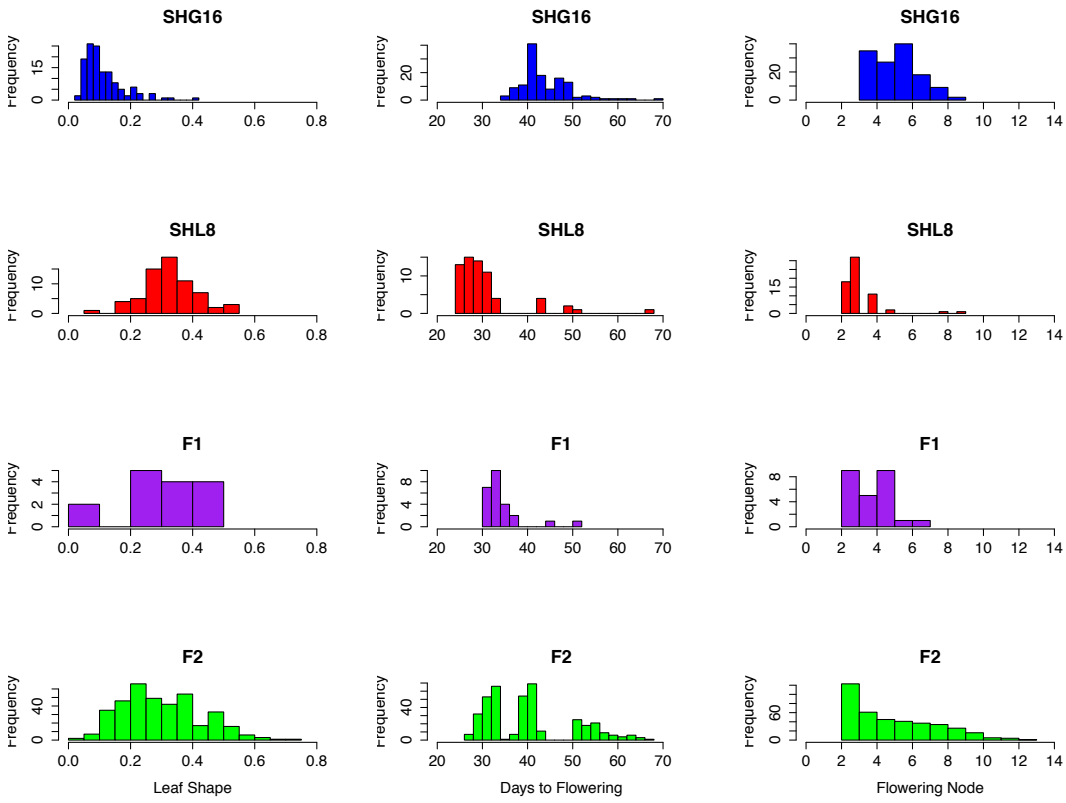

Figure 15. Phenotypic distributions of leaf shape, days to flowering, and flowering node for M. guttatus (blue), M. laciniatus (red), F1 (purple), and F2 (green) populations.

parental line seeds were cold stratified for ten days and then placed in the greenhouse to germinate. Leaf shape was measured on the day of first flower by taping leaves to sheets of white paper, digitally scanning them and doing a convex hull analysis of each leaf image in ImageJ. Convex hull analysis consists of connecting the outermost points of a leaf to create an entire leaf shape. The true area of the leaf was subtracted from the area of the convex hull. Then the difference in area between these two shapes was divided by

Table 10. Phenotypic correlation matrix among all F2 individuals in our common garden experiment.

	Shape	Days to Flow	Flower Node	Corolla W	Corolla L (u)	Corolla L (l)
Shape	1					
Days to Flow	0.4311	1				
Flower Node	0.4343	0.8846	1			
Corolla W	0.3516	0.6403	0.7411	1		
Corolla L (u)	0.2471	0.4502	0.5414	0.7663	1	
Corolla L (l)	0.3654	0.6461	0.7411	0.9438	0.8433	1

the area of the convex hull to control for leaf size. Flower size was measured on the day of first flower in three dimensions: corolla width, corolla length upper, and corolla length lower (see Fishman et al 2002 for diagram). Each of these measurements was performed with a small metal ruler and measured in 100ths of an inch. Flowering time was measured as number of days between seeds being placed in the greenhouse post-cold stratification and the day of first flower. Leaf and bud tissue was collected from each F2 in the mapping population and frozen at -80 degree C before DNA extraction.

4.3.3 QTL Mapping Approach

In order to map quantitative trait loci (QTL) involved in the ecologically relevant traits of flowering time, leaf shape, and flower size we used the multiplexed shotgun genotyping (MSG) approach developed by the Andolfatto lab at Princeton University (Andolfatto et al 2012). This powerful QTL mapping method uses restriction enzyme digestion, barcoding and next generation sequencing to create many single nucleotide polymorphism markers across the entire genome. Barcoding each DNA sample allows

multiplexing of many F2 or other individuals in one lane of Illumina sequencing which makes it cost effective as well as powerful.

In our experiment DNA was extracted from each of the 1000 F2's in our SHL8 x SHG16 mapping population using a modified CTAB protocol (Kelly and Willis 1998). The DNA concentration of each F2 sample was quantified using Quant-IT picogreen and a microplate reader. An equal amount of DNA from each of 424 SHL8 x SHG16 F2s was digested with the restriction enzyme MSE1 for 3 hours at 37C. Then 9 sets of 48 barcoded adaptors were ligated onto the ends of the digested DNA samples. We then pooled each set of 48 barcoded samples to make 9 libraries. Libraries were cleaned with Agencourt Ampure bead purification and then size selected to contain fragments between 250-400bp using gel extraction. After size selection 9 different Illumina FC1 and FC2 adaptors were added to the libraries by amplifying barcoded fragments with the Phusion PCR kit. The final libraries were bead purified again with a bead purification step. DNA concentration of each library was determined using a Qubit® fluorometer. Finally, equal amounts of each library were combined into one sample for Illumina Sequencing.

Our multiplexed sample of 424 F2's was sequenced on an Illumina Hiseq in two lanes of with 50bp single end reads. We got 550 million reads total. The average coverage for each individual F2 was 0.15X. To map QTLs using our MSG generated data

we used a bioinformatics pipeline composed of TASSEL v.3.0 (Glaubitz et al in press) and Bowtie v.2.1.0 (Langman and Salzberg 2012) to align our short read data to the IM62 *M. guttatus* genome and call SNPs. By creating bins of 75 SNP markers a haplotype map was generated across the entire genome for each F2 individual. The genotype matrix that was output by this bioinformatics pipeline was then analyzed in Rqtl (Broman et al. 2003). QTL's were identified using interval mapping. LOD score significance threshold was determined using a permutation test. QTL effect size was measured in several ways, first by the R^2 term from a one-way analysis of variance (ANOVA), and second by calculating the proportion of the mean parental difference in a trait that each QTL explained. The presence of epistasis between QTLs was detected by looking for significant interaction terms in a factorial ANOVA.

4.3.4 Phenotypic Selection in the Field

In order to test whether leaf shape, flowering time, and flower size were adaptive in *M. laciniatus's* granite outcrop habitat and whether *M. laciniatus* and *M. guttatus* are locally adapted we performed a reciprocal transplant experiment using the *M. laciniatus* inbred line WLF47, the *M. guttatus* inbred line YVO6, and WLF47xYVO6 F4 hybrids. Outbred F4 hybrids were created by first self-fertilizing F1 hybrids to generate an F2 population. Two generations of random mating among 200 maternal families proceeded to create a population of randomly intercrossed F4 seeds. We did not keep track of

maternal family in our field experiment because of logistical challenges so we pooled ~30 seeds from each maternal plant to create one large outbred F4 family.

In April of 2013 a total of 400 WLF47, 400 YVO6, and 3000 F4's were planted in open flats of soil in the UC Davis greenhouses. Seeds were cold stratified for 10 days and then left in the greenhouse to germinate for one week. Seedlings were transplanted one inch apart into 50 randomized blocks of 19 plants (2 WLF47, 2 YVO6, 15 F4) at the cotyledon stage at each of four field sites off of Tioga Road in Yosemite National Park. The *M. laciniatus* habitat field sites, Olmstead Point (OP) and Yosemite Creek (YC), are undisturbed granite outcrops with native *M. laciniatus* populations growing on moss at elevations of 8,500 and 7,500 feet respectively The *M. guttatus* habitat field sites, Little Meadow (LM) and Crane Flat, are both undisturbed open moist meadows with native *M. guttatus* populations growing in and around a standing seep. They occur at 6,200 and 6,000 feet respectively. These sites were chosen so that seedlings and native plants would be at similar stages.

Plants were censused every other day from May through August of 2013 for timing of the first flower and survival. On the day of first flower date of first flower, corolla width, and plant height were measured. The first flower from each plant that survived to flowering was collected and placed in silica gel to dry for DNA extraction. Morphological measurements were taken with a small metal ruler in 100ths of an inch.

Table 11. QTL position, significance level and effect size along with the mean phenotype of the M. guttatus homozygote (GG), the heterozygote (GL), and the M. laciniatus homozygote (LL) are described in the table below. LOD score and the F-ratio from one-way ANOVAs are given to describe the significance level of the QTL association with phenotype. Both R^2 and the proportion of the mean parental difference in phenotype are given to describe effect size. The mean phenotype for each genotype was determined in a one-way ANOVA at each locus. Significance levels are represented as p-value <0.1 = *, <0.05 = **, <0.01 = *, <0.001 = ****.**

Trait	Heritability	Chromosome	Position (bp)	LOD Score	F Ratio	R^2	Porportion Parental Difference	Mean GG Phenotype	Mean GL Phenotype	Mean LL Phenotype	QTL Direction
Corolla Length Lower	0.2897136	8a	8475937	8.36	13.22****	0.097	0.248587551	85.5	78.51	66.86	+
		8b	18587008	15.99	25.88****	0.173	0.26725829	85.53	78.98	65.49	+
		10a	3606370	6.31	8.51****	0.065	0.184973677	83.54	75.89	69.67	+
		10b	9388712	7.69	12.34****	0.091	0.229516725	84.83	76.77	67.62	+
		8a x 8b			1.87*	0.24					
		8b x 10b			1.71*	0.27					
Corolla Length Upper	-0.1198038	8a	4821666	5.88	8.01****	0.061	0.174160536	55.13	52.69	47.74	+
		8b	16535486	5.92	11.02****	0.082	0.194428203	56.16	51.92	47.91	+
		10a	3606370	7.63	10.82****	0.081	0.198434603	56.23	51.39	47.81	+
		10b	9388712	6.56	8.65****	0.066	0.177931265	55.04	52.13	47.49	+
		11	2917774	4.52	8.5****	0.064	0.140930989	53.53	52.89	47.55	+
Corolla Width	0.247763	8a	6859184	7.97	14.62****	0.106	0.253575132	66.09	59.49	47.63	+
		8b	18587008	16.83	22.01****	0.151	0.278300768	66.7	58.92	46.44	+
		10a	3606370	5.43	8.99****	0.068	0.171293711	64.23	56.74	51.76	+
		10b	9388712	6.44	8.99****	0.068	0.171293711	64.23	56.74	51.76	+
Flowering Node	0.739086	8a	8475937	7.23	17.72****	0.12	1.360276641	7.65	5.69	4.37	+
		8b	18587008	24.87	43.46****	0.251	1.613254919	7.51	5.8	3.62	+
		10a	1981685	5.67	9.68****	0.069	0.862614455	6.53	5.68	4.45	+
		10b	9388712	6.53	15.46****	0.107	1.144623027	6.84	5.66	4.08	+
		8a x 10b			1.91**	0.242					
		8b x 10a			2.10**	0.326					
Flowering Time	0.4733213	7	1176068	4.22	11.43****	0.021	0.358473885	37.9	43.73	42.65	-
		8a	7343804	7.4	17.44****	0.119	0.808264276	48.27	41.4	37.56	+
		8b	18362020	25.8	48.59****	0.273	1.140324296	49.66	42.26	34.55	+
		10a	1981685	5.7	9.86****	0.071	0.591670581	45.39	42.03	37.55	+
		10b	6802769	7.21	16.53****	0.113	0.887505872	49.46	41.07	37.7	+
		8b x 7			2.09**	0.342					
		8b x 10a			2.41**	0.321					
		8b x 10b			1.72*	0.352					
Leaf Shape	0.4025274	2	17309441	5.36	10.99****	0.081	0.611759103	0.253	0.304	0.375	+
		5	1281889	3.37	6.28*	0.048	0.496427469	0.345	0.311	0.246	-
		8a	3909698	3.93	8.56****	0.064	0.601730265	0.386	0.312	0.266	-
		8b	19759651	7.62	12.81****	0.093	0.631816779	0.368	0.317	0.242	-
		11	9374956	3.53	5.91**	0.045	0.471355375	0.369	0.309	0.275	-

Leaf shape was not measured at this time because of fear of imposing selection by damaging plants before they had begun to set seed. Leaves were collected for analysis when plants first showed signs of senescence at each site. Leaf area and degree of lobing were determined by digitally scanning leaves taped to a white piece of paper and then performing convex hull analysis as described previously. Fruit number was counted once plants had senesced as a measure of lifetime fitness. Fruits were collected in order to someday count seeds for each individual. In the Little Meadow population we were not able to collect fitness data for ~50% of the plants due to our experimental site burning in the Yosemite Rim Forest Fire of 2013 before the final fitness census could be conducted.

To look for genotype by environment interactions we measured trait means and standard errors in each transplant population separately for F4, *M. laciniatus,* and *M. guttatus* individuals. We also measured phenotypic correlations among traits for each habitat type (granite vs. meadow) using a restricted maximum likelihood model (REML) in JMP Pro10. To test for local adaptation between *M. laciniatus* and *M. guttatus* we conducted a logistic regression in R using survival to flowering as the dependent variable and species, habitat and the interaction between species and habitat as the independent variables. We also plotted percent survival to flowering and meant fruit number for each species in granite vs. meadow habitat and looked for crossing reaction

norms. And finally we used two tailed t-tests to determine whether mean survival to flowering and fruit number were significantly different between *M. laciniatus* and *M. guttatus* in each habitat. A Bonferroni correction was used to control for multiple comparisons (α=0.025).

To look at phenotypic selection in native habitats we conducted linear and quadratic selection analysis (Lande and Arnold 1983, Mitchell-Olds and Shaw 1987) by regressing fruit number (fitness) simultaneously on flowering time, corolla width, leaf area, and leaf shape in our experimental F4 population. We used a zero-inflated poisson model on the individual phenotypes to conduct our regression analysis (as in Anderson et al 2012). Block was included as a fixed effect due to the difficulty of programming it as a random effect in R package pscl. We did not include height at first flower in this analysis because it was positively correlated with flower width and leaf area in both habitats (Table 14). Zero-inflated poisson models control for excess zeros in count data by simultaneously breaking up the data into a logistic regression on in our case whether a plant set seed or not, which has a binomial distribution, and performing a poisson regression on the count data for plants that did set seed (Ridout et al 1998). All phenotypes were standardized to have a mean of 0 and standard deviation (s.d.) of 1 to enable comparison of traits measured in different units.

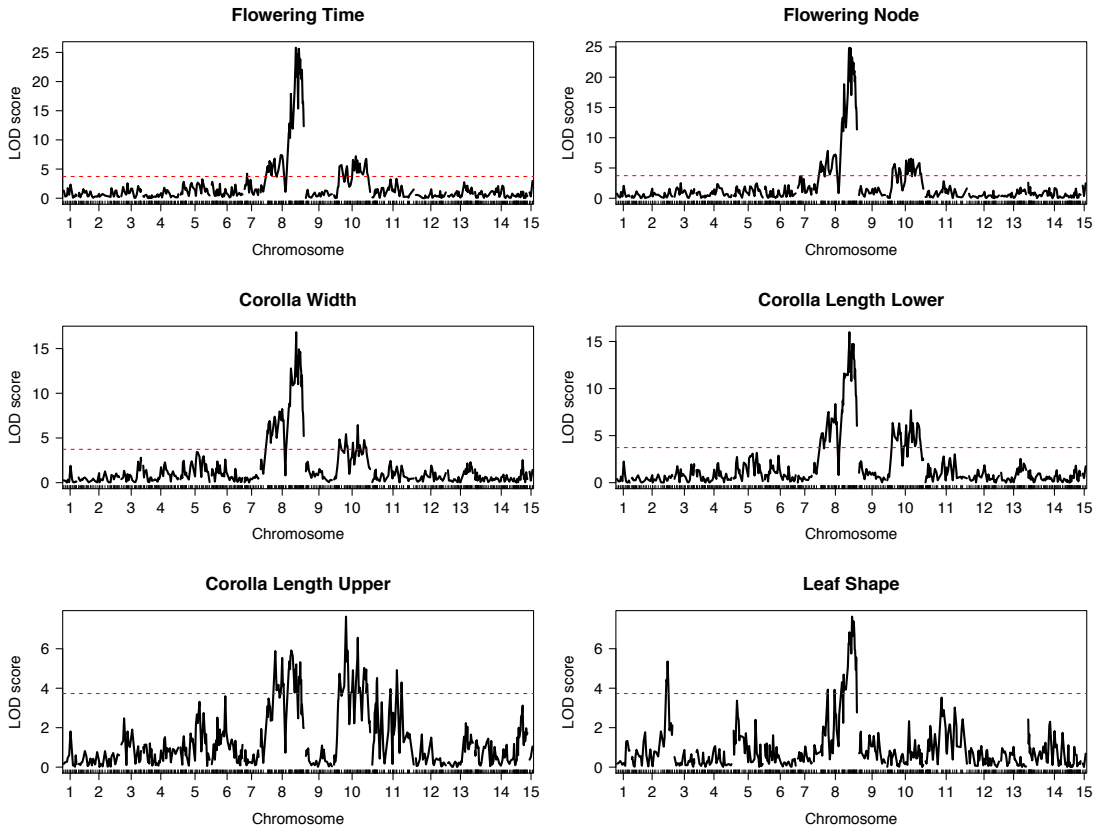

Figure 16. QTL maps for all six phenotypes measured in our common garden experiment: flowering time, node of first flower, corolla width, corolla length lower, corolla length upper, and leaf shape. LOD score significance threshold is indicated by the dotted red line.

4.3.5 Fitness QTL Mapping in the Field

As another way of determining whether flowering time, flower size, and leaf shape are related to fitness in these species' natural habitats we tested whether QTL's from our common garden experiment in the Duke Greenhouses were involved in fitness in the

field. By looking at the relationship between QTL genotype and fitness in each habitat we can also test whether antagonistic pleiotropy or conditional neutrality underlies local adaptation between *M. laciniatus* and *M. guttatus* at the individual locus level.

In order to do this we extracted DNA from dried floral tissue from each F4 plant that survived to flowering in all four of our transplant sites for a total of 563 samples (273 from OP, 122 from YC, 45 from LM, and 123 from CF). We genotyped each of these F4's at a single exon-primed intron crossing (EPIC) marker derived from expressed sequence tags (ESTs) (Fishman et al 2008), *MgSTS538*, beneath a large effect pleiotropic QTL on the right arm of chromosome 8 (LG8b) from our common garden mapping experiment. This QTL contributed to species differences in flowering time, flower size, node of first flower, and leaf shape in the greenhouse and had a large effect on each trait (Table 11). Because of its effect size and pleiotropic nature we hypothesized that of all our common garden QTL's, LG8b was the most likely to be related to fitness in a field study.

We screened parental inbred lines for polymorphism at *MgSTS538*. Polymorphism was determined by variation in PCR fragment length, which is usually due to insertion/deletion (indel) variation in introns. Primers for these markers can be found on the Mimulus Evolution website (http://www.mimulusevolution.org). PCR products underwent capillary electrophoresis and fragment analysis on an ABI 3730xl

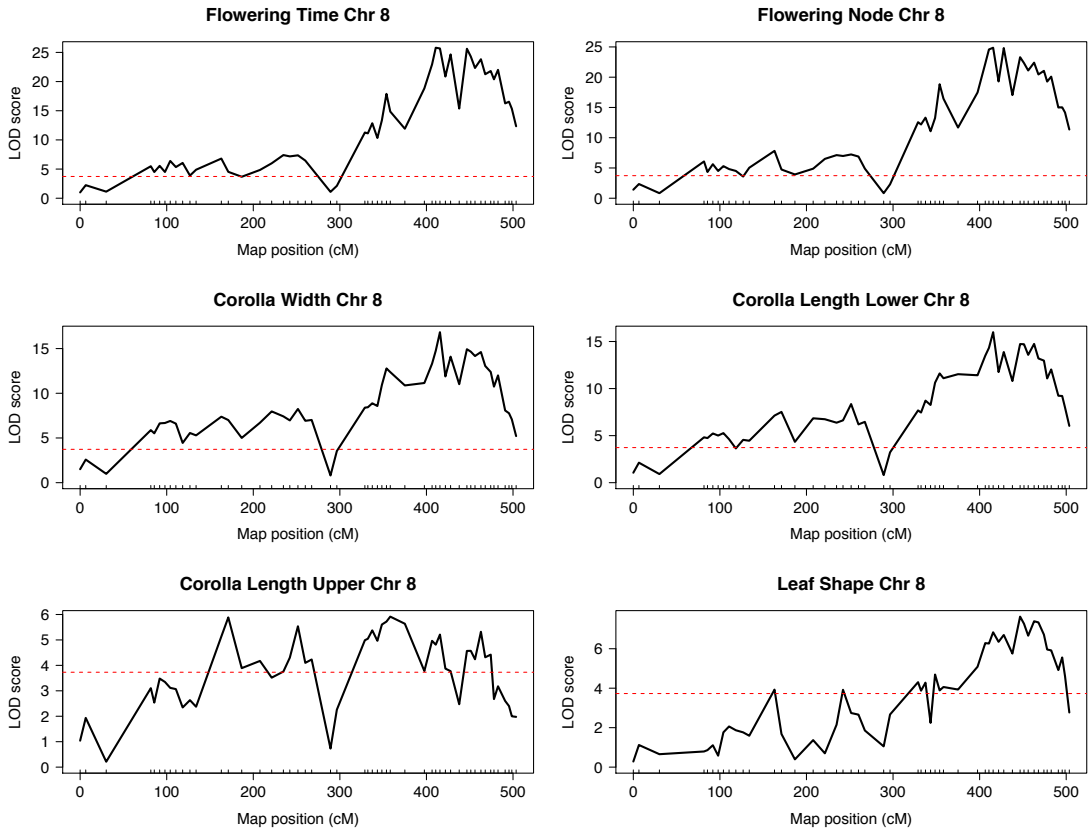

Figure 17. Plots of LOD score along chromosome for flowering time, node of first flower, corolla width, corolla length lower and upper, and leaf shape. Map position along the x-axis is based on a physical map of the M. guttatus genome and not actually in cM.

DNA Analyzer (Applied Boisystems, Foster City, CA, USA). Fragment size was scored in the program GeneMarker (Soft Genetics, State College, PA, USA.). To test for an association between LG8b QTL genotype and fecundity we performed a one-way analysis of variance (ANOVA) using R with fruit number as our dependent variable and

MgSTS538 genotype as our independent variable. We analyzed our genotype data for each of our four reciprocal transplant sites separately. To test whether there was selection on LG8b before flowering we used a chi-squared test to look for skew in the genotype ratios among plants that survived to flowering at each transplant site. Under no selection we expect the genotypic ratios in each site to be 1:2:1 (*M. laciniatus* Homozygote : Heterozygote : *M. guttatus* Homozygote). However, if there was selection at this locus before flowering then our observed genotypic ratios should significantly differ from our expected ratio. To correct for multiple tests across population sites in both our ANOVA and chi-squared analyses we used the Bonferroni correction and divided our significance level $\alpha = 0.05$ by the number of tests to get $\alpha = 0.0125$.

4.4 Results

4.4.1 Species differences genetically based and simple

From our common garden experiment in the Duke University greenhouse we see that the *M. guttatus* parent SHG flowers later, and at a later node than the *M. laciniatus* parent SHL (Figure. 15). Also that SHG has larger flowers than SHL in terms of all three corolla size measurements: width, length lower, & length upper (Figure 14) and that SHL possesses more highly lobed leaves than SHG. We know that these phenotypic differences are genetically based because they appear even in a common environment. The phenotypic distributions of all 6 traits indicate that they are genetically simple

because the parental trait distributions largely overlap the F2 distribution for each trait (Figures 14&15). All six traits were positively correlated (Table 10), but the strongest correlations were between flowering time and node of first flower (corr = 0.88) and corolla width and corolla length lower (corr = 0.94). Lobed leaf shape was positively correlated with both flowering time and flower size which was unexpected given the parental distributions of these traits (Table 10, Figures 14 & 15).

4.4.2 Common Garden QTL Mapping Results

In order to examine the genetic basis of putatively adaptive phenotypic differences between *M. laciniatus* and *M. guttatus* we mapped quantitative trait loci (QTL's) involved in flowering time, node of first flower, flower size, and leaf shape in the SHG x SHL F2 population in a common garden experiment. Using interval mapping we found a single large effect QTL (R^2 = 0.08-0.27) on the right arm of chromosome 8 (LG8b) that was common to all six traits: leaf shape, flowering time, node of first flower, corolla width, corolla length lower, and corolla length upper (Table 11, Figures 16 & 17). There was also a significant QTL on the left arm of chromosome 8 (LG8a) of smaller effect (R^2 = 0.06-0.12) that was common to all traits. Two QTL's of moderate effect on LG10, one on the left arm (LG10a, R^2 = 0.06-0.08) and one on the right (LG10b, R^2 =0.068-0.11), were common to all of our traits except leaf shape.

Table 12. Phenotypic trait means and standard errors calculated for *M. laciniatus's* granite outcrop versus *M. guttatus's* meadow habitat type in F4's, *M. laciniatus*, and *M. guttatus*.

Plant	Trait	Mean Granite	SE Granite	Mean Meadow	SE Meadow
F4	Days to 1st Flower	77.85	0.263	86.42	0.774
	Height (100ths of ")	162.18	3.154	180.72	7.054
	Flower Width (100th	33.8	0.524	25.17	0.984
	Leaf Area (megapixel	2009.09	157.565	3202.73	281.15
	Leaf Shape	0.13	0.002	0.12	0.004
M. laciniatus	Days to 1st Flower	76.96	0.505	85.78	1.327
	Height (100ths of ")	114.36	4.356	186.13	19.062
	Flower Width (100th	24.3	1.012	15.5	1.867
	Leaf Area (megapixel	1068.4	143.958	1306.54	248.161
	Leaf Shape	0.18	0.011	0.17	0.018
M. guttatus	Days to 1st Flower	80.25	0.743	82.3	1.739
	Height (100ths of ")	124.93	6.668	297.84	28.129
	Flower Width (100th	35.2	1.68	39.34	1.89
	Leaf Area (megapixel	1120.42	103.143	3524	647.07
	Leaf Shape	0.11	0.006	0.11	0.01

We also found unique QTL's. One QTL was unique to upper corolla length on chromosome 11 (LG11, R^2=0.06) and a QTL on chromosome 7 was unique to flowering time (LG7, R^2=0.02). We found a large effect QTL that was unique to leaf shape on the right arm of chromosome 2 (LG2, R^2=0.08) and two smaller effect unique leaf shape QTLs: one on the left arm chromosome 11 (LG11, R^2=0.4) and one on the right arm of chromosome 5 (LG5, R^2=0.05). The QTL's on chromosomes 2 and 11 overlap with those found in our previous study of leaf shape in a different *M. laciniatus* x *M. guttatus* cross (see Chapter 3). The leaf shape QTLs on chromosomes 8 and 5 do not overlap with our previous findings.

Table 13. Phenotypic trait means and standard errors (SE) calculated for each reciprocal transplant site (OP, YC, LM, CF) separately in F4's, *M. laciniatus,* and *M. guttatus*. OP stands for Olmsted Point and is granite outcrop habitat, YC stands for Yosemite Creek and is also granite outcrop habitat, LM stands for Little Meadow and is meadow habitat, CF stands for Crane Flat and is also meadow habitat.

Plant	Trait	Mean OP	SE OP	Mean YC	SE YC	Mean LM	SE LM	Mean CF	SE CF
F4	Days to 1st Flower	80.13	0.305	73.81	0.313	86.42	0.774	89.85	0.654
	Height (100ths of ")	153.98	3.836	176.84	5.327	180.72	7.054	353.8	13.88
	Flower Width (100th	35.14	0.675	31.14	0.766	25.17	0.984	27.64	1.011
	Leaf Area (megapixel	1326.37	54.367	2686.67	311.833	1308.98	77.038	3899.36	264.839
	Leaf Shape	0.12	0.0017	0.12	0.049	0.12	0.0029	0.12	0.0036
M. laciniatus	Days to 1st Flower	77.91	0.656	74.83	0.551	84.26	1.754	87.82	1.98
	Height (100ths of ")	109.51	4.493	126.12	9.974	133.23	12.4	258.88	34.976
	Flower Width (100th	25.53	1.131	20.28	2.009	16.69	2.676	14.31	2.671
	Leaf Area (megapixel	915.31	104.856	2109.56	992.606	1022.12	209.607	1741.77	284.819
	Leaf Shape	0.166	0.0097	0.22	0.018	0.13	0.013	0.18	0.019
M. guttatus	Days to 1st Flower	82.03	0.671	75.31	1.283	82.76	2.078	93	2.233
	Height (100ths of ")	115.22	6.623	150.83	14.959	202	19.821	376.76	39.753
	Flower Width (100th	35.29	1.913	34.9	3.704	34.43	2.528	43.41	2.387
	Leaf Area (megapixel	888.12	79.429	1497.79	185.244	1490.35	214.149	3834.51	413.18
	Leaf Shape	0.1	0.0047	0.1	0.008	0.11	0.0135	0.1	0.0075

We found evidence for epistasis between QTL's for flowering time, flower node, and lower corolla length (Table 11). All of these significant interaction terms in our ANOVA models were between our pleiotropic QTL's on chromosomes 10 and 8. Several of our QTL's are negative in direction. The QTL on chromosome 7 for flowering time is negative meaning that *M. guttatus* genotypes at this QTL flowered earlier than *M. laciniatus.* More surprisingly all QTL's for leaf shape, except for the one on chromosome 2, are negative in direction (Table 11). This means that at four of our five leaf shape QTL *M. guttatus* genotypes have more highly lobed leaves than *M. laciniatus* genotypes.

4.4.3 Local adaptation and phenotypic selection in granite outcrops

In order to understand how ecologically important traits vary between our habitats and species we calculated the mean phenotypic value and standard error for

flowering time, plant height, flower width, leaf area, and leaf shape for F4's, *M. laciniatus* (WLF47), and *M. guttatus* (YVO6) separately by habitat (granite vs. meadow) and for each separate population site (OP, YC, LM, CF). We see evidence of plasticity in all five traits. Within each genotypic class (F4, *M. laciniatus, M. guttatus*) plants flowered earlier, were shorter, and had smaller leaves in the *M. laciniatus* granite outcrop habitats than they did in the *M. guttatus* meadow habitats (Table 12). This plasticity is in the direction that we would expect.

Surprisingly in the F4s and *M. laciniatus,* flowers were larger on average in the granite outcrop habitat than in the meadow habitat. In *M. guttatus* this trend has in the expected direction though, with larger flowers occurring in the meadow habitat. There was not much difference in leaf shape between the two habitats, but when we looked at individual population means there was variation in *M. laciniatus* leaf shape across individual population sites (Table 13). We also see differences between genotypic classes in flowering time within populations, with *M. laciniatus* flowering earlier than nearby *M. guttatus,* except in the Little Meadow population where the trend is reversed.

We also looked at phenotypic correlations among traits in each environment (Table 14). In both environments we see that leaf area, flower size, and height at first flower are highly positively correlated (corr > 0.5). In the granite habitat flowering time is slightly negatively correlated with height (-0.073), leaf area (-0.088), and leaf shape

Table 14. Phenotypic correlation matrix among all traits at granite vs. meadow sites separately. The granite habitat correlations are in the bottom matrix and the meadow habitat correlations are on the top.

	Days to Flowering	Height	Flower Width	Leaf Area	Shape
Days to Flowering	1	0.1098	0.0226	0.1284	-0.0582
Height	-0.0725	1	0.5565	0.5341	0.0101
Flower Width	0.0468	0.677	1	0.3051	0.0002
Leaf Area	-0.0883	0.5254	0.3403	1	0.1355
Shape	-0.0521	0.0456	0.0801	0.0797	1

(-0.0521). In contrast, in the meadow habitat flowering time is positively correlated with height (0.110) and leaf area (0.128). Leaf area and shape are slightly positively correlated in each habitat (granite= 0.079, meadow = 0.136).

To look for local adaptation of our parental lines to granite versus meadow habitats we ran a logistic regression of survival to flowering on the interaction between species and habitat (Table 15). We find that there is a significant interaction between habitat and species. To visualize this data and look for crossing reaction norms we plotted proportion surviving to flower for each species in each habitat (Figure 18). We also performed two tailed t-tests to see how mean survival to flowering and fruit number differed between *M. laciniatus* and *M. guttatus* in each habitat. In the granite habitat *M. laciniatus* has a significantly higher survival rate than *M. guttatus* (0.23 > 0.12, p-value < 0.0001). In contrast, both species have highly similar survival rates in the *M. guttatus* meadow habitat (*M. guttatus* = 0.09, *M. laciniatus* = 0.10, p-value = 0.72). These data indicate that there is no trade-off in survival to flowering between the two species'

Table 15. Results of a logistic regression of survival to flowering on species and habitat to test for local adaptation. The model was Survival to Flowering ~ Habitat (Granite or Meadow) + Species (M. guttatus or M. laciniatus) + Habitat*Species.

Independent Variables	β'	z-value	p-value
Species(L)	0.092	0.356	0.722
Habitat(L)	0.265	1.071	0.284
Species(L)*Habitat(L)	0.964	2.876	0.004**

habitats since *M. laciniatus* performs just as well as *M. guttatus* in the meadow habitat. However when we plot mean fruit number for each species in each habitat we do see a suggestion of a fitness trade-off due to crossing reaction norms (Figure 18). *M. laciniatus* has significantly higher mean fruit number in the granite habitat (mean = 0.48, SE = 0.05) than *M. guttatus* (mean = 0.10, SE = 0.022, p-value <0.0001), while *M. guttatus* has slightly higher mean fruit number in the meadow habitat (*M. guttatus* = 0.05, SE = 0.022, *M. laciniatus* = 0.007, SE = 0.007). However, once we have corrected for multiple comparisons the difference in fruit number is not significant in the meadow habitat (p-value = 0.055, $\alpha = 0.025$).

In order to assess selection on individual phenotypic traits in granite and meadow habitats we performed linear and quadratic selection analysis by performing a zero-inflated poisson regression of fruit number on flowering time, flower width, leaf area, and leaf shape in our experimental F4 population. In the poisson regression portion of the model, which tested for correlations between phenotypic value and fruit number among plants that did set seed, we found significant directional and quadratic selection

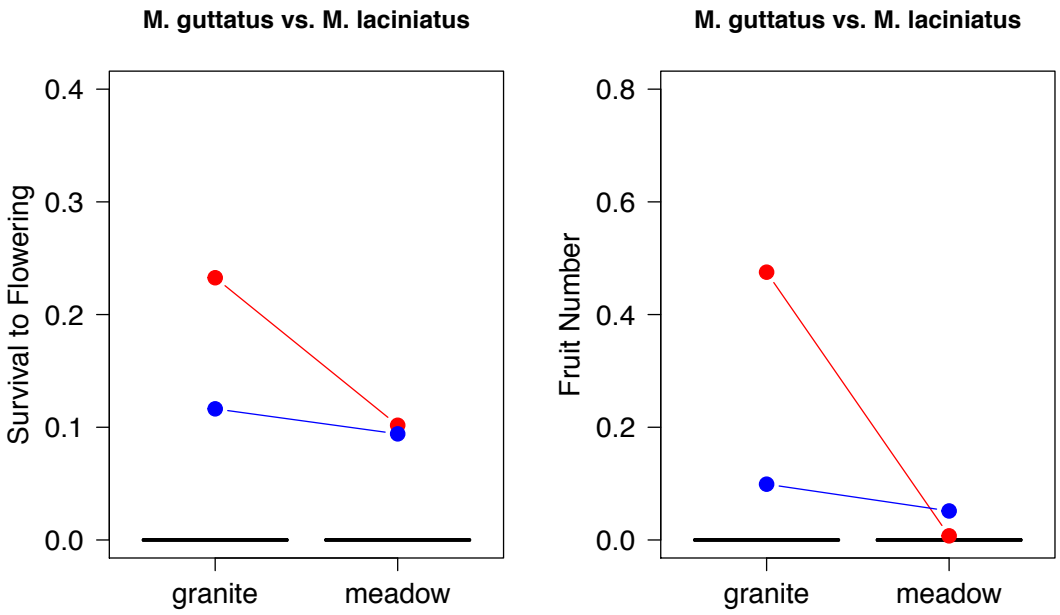

Figure 18. Reaction norms for *M. laciniatus* (red) and *M. guttatus* (blue) for survival to flowering and fruit number between granite and meadow habitats.

coefficients. There was directional selection for earlier flowering in both habitats, but only significant directional selection on earlier flowering in the granite habitat (Table 16). There was significant directional selection for increased flower width in both habitats. There was no significant directional selection on leaf area or leaf shape across habitats, but there was significant negative quadratic selection on leaf area in the granite habitat and significant negative quadratic selection on leaf shape in the meadow habitat (Table 16). However we did find significant positive directional selection on leaf shape

in the Crane Flat meadow, which is in the opposite direction that we expected. To test whether selection was operating differently on traits between habitats and populations we also looked for an interaction between each trait & habitat type and each trait & population. We found two significant interactions, one between flowering time and habitat type and one between flowering time and population (Table 16).

In the logistic regression portion of our zero-inflated Poisson model we tested whether phenotypic values were correlated with the probability that an individual F4 set seed or not. In this portion of the test we found significant positive selection on leaf area in the meadow habitat and significant negative quadratic selection on leaf area in the Olmsted Point and Little Meadow population populations. We also found significant interactions between population and leaf area (Table 17). We recognize that all of these selection gradient values could be due to selection on unmeasured correlated characters.

4.4.4 Does our Pleiotropic Greenhouse QTL Affect Fitness in the Field?

The results of our genotypic analysis in the field are mixed. After correcting for multiple tests we found no significant effect of LG8b genotype on fruit number in any of our reciprocal transplant sites (Table 18). Even though we did not find a significant effect of genotype on fecundity in the either of the granite outcrop sites, when we look at our genotype vs. fruit number plots we see that in the Yosemite Creek granite site *M.*

Table 16. Results of phenotypic selection analysis on flowering time, flower width, leaf area, and leaf shape using a zero-inflated poisson regression. This table contains the regression coefficients from the poisson regression portion of the model on fruit count. β represents the selection gradient for each phenotypic trait in each habitat type and population. Dashes represent missing or non-significant data. Significant codes are as follows: p-value < 0.001 = *, 0.01= **,0.05 =* .**

Trait	β Granite	β Meadow	β OP	β YC	β LM	β CF
Days to 1st Flower	-0.857***	-0.2464	-0.8474***	-1.2115***	-0.454	-0.4548**
Flower Width (100ths of ")	0.15*	0.3796*	0.1226	0.1821	0.1091	0.5075**
Leaf Area (megapixels)	0.0522	0.4077	0.2471	0.0849	-	0.4062
Leaf Shape	0.0541	0.2545	0.0605	-0.0419	-1.0089	0.3887*
Block	-0.0037	-0.0303*	0.0043	-0.0062	0.0199	-0.0381**
Leaf Area non-linear (γ')	-0.0162*	-	-0.3966*	-	-	-
Leaf Shape non-linear (γ')	-	-0.3793**	-	-	-	-0.3356*
Flowering Time (γ')	-	-	-	-	-	-0.3422*
Days to 1st Flower*Habitat	-3.911e^-1**	-	-	-	-	-
Days to 1st Flower*Population	-	-	-0.6689***	-0.4154	-0.0166	-

laciniatus homozygotes at LG8b have higher fruit number than *M. guttatus* homozygotes (Figure 19). This trend is not significant due to our small sample size from Yosemite Creek, especially because of the small number of *M. laciniatus* homozygotes that we sampled (Figure 20). The site with the largest number of genetic samples was the *M. laciniatus* site Olmsted Point and here we see no relationship between LG8b genotype and fruit number (Figure 19). At Crane Flat both heterozygotes (GL) and *M. laciniatus* homozygotes (LL) produced more fruits than *M. guttatus* homozygotes (GG) and this trend would have been significant if not for our Bonferroni correction (Figure 19). This pattern is in the opposite direction of what we would expect given our phenotypic selection analysis and common garden QTL mapping experiment.

To test whether there was selection before flowering at the LG8b locus we looked for a skew in the genotype ratios at each transplant site in our sample of plants that survived to flowering. If there was no selection then the genotypic ratios in each site should be 1:2:1 (LL:GL:GG), however if there was selection at this locus before flowering then our genotypic ratios should differ from 1:2:1. After correction for multiple testing our chi-square test results were significant at two of the four transplant sites: the granite site Olmsted Point and the meadow site Crane Flat (p-values: YC=0.45, OP < 0.001**, LM=0.056, CF<0.001**). Our finding indicates that in OP and CF, our two experimental populations with the highest number of genetic samples, the genotype frequencies were significantly skewed. The Yosemite Creek population had the fewest genetic samples and did not have a significant X^2-value, but when we plot the genotype frequencies it looks like there is an excess of heterozygotes at this site (Figure 20). There appears to be an excess of *M. guttatus* homozygotes (GG) at both meadow sites (Little Meadow, Crane Flat) and the granite Olmsted Point site as well (Figure 20).

4.5 Discussion

4.5.1 Large Effect Pleiotropic QTL Controls Species Differences

In our common garden QTL mapping experiment we found that all six of our morphological and life history traits had a relatively simple genetic basis (4-5 QTL per trait). This genetic simplicity is surprising for the floral size traits we measured since in

Table 17. Results of phenotypic selection analysis on flowering time, flower width, leaf area, and leaf shape using a zero-inflated poisson regression. This table contains the regression coefficients from the logistic regression portion of the model on whether plants set seed or not. β represents the selection gradient for each phenotypic trait in each habitat type and population. Significant codes are as follows: p-value < 0.001 = *, 0.01= **,0.05 =* .**

Trait	β' Granite	β' Meadow	β' OP	β' YC	β' LM	β' CF
Days to 1st Flower	-381.856	-0.2617	1.569	-196.15	-9.5316	-3.959
Flower Width (100ths of ")	243.902	-0.6145	-0.2416	225.66	0.07021	0.939
Leaf Area (megapixels)	-534.315	4.0554*	1.9675	-280.893	-	4.76*
Leaf Shape	-54.192	0.0765	0.1158	-41.99	-34.667	1.665
Block	1.492	-0.2049	0.3511**	-1.567	0.62198	-0.383*
Leaf Area non-linear (γ')	-	-	-3.1**	-	-	-
Leaf Area*Population	-	-	1.9032*	-3.0741	10.1948*	-

former studies of both intra and interspecific floral differences in *Mimulus* flower size was controlled by many (16-20) QTL's of small effect (Fishman and Willis 2002, Hall et al 2006). We also found that interspecific differences in flowering time, flower size, node of first flower, and leaf shape were largely controlled by a single large effect QTL. This pleiotropic QTL, LG8b, explained the largest proportion of the variance in the F2 population of all six characters we measured in our common garden experiment. We define a pleiotropic QTL as a genomic region that effects multiple traits. We do not know whether this region consists of a single truly pleiotropic locus or many tightly linked loci. Three other pleiotropic QTL's were also found in our analysis: LG8a was common to all six traits, and LG10a & LG10b contributed to differences in flowering and floral size traits, but not to leaf shape. Leaf shape contained three unique QTL's. The one

on LG2 overlapped with a QTL in our bulk segregant leaf shape mapping experiment in a different cross (see Chapter 3) and was of equally large effect as the pleiotropic LG8b. Previous studies have found a major pleiotropic QTL controlling life history and morphological characters between inland and coastal forms of *M. guttatus* (Hall et al 2006). This pleiotropic QTL was also on chromosome 8 and turned out to be a widespread chromosomal inversion between the two forms of *M. guttatus* (Lowry and Willis 2010). Our large effect pleiotropic QTL, LG8b, is on the opposite end of the chromosome from this inverted region within *M. guttatus*, but LG8a is in the same area. It is difficult to tell from our current analysis whether LG8b is in an inverted region. The QTL region is large for LG8b, but there is a definite peak in LOD scores so that it does not resemble an area of uniformly suppressed recombination. We also did not use traditional pcr-based markers whose genomic location is known in *M. guttatus* in our mapping experiment, but instead used bins of 75 single nucleotide polymorphism (SNP) loci along the entire genome as genetic markers. Thus we cannot tell whether marker order is reversed between the parents of our cross within the QTL region. It would be necessary to genotype our parents and F2's at several traditional *M. guttatus* pcr-based markers within LG8b to determine whether this pleiotropic locus was in fact within an inversion.

Table 18. One-way analysis of variance (ANOVA) table with fruit number as the dependent variable and genotype at LG8b as the independent variable. Genotype has three levels: GG (M. guttatus homozygote), GL (heterozygote), and LL (M. laciniatus heterozygote). We report the degrees of freedom (Df), sum of squares (Sum Sq), mean square (Mean Sq), F-statistic (F-value) and p-value for each reciprocal transplant site: Yosemite Creek, Olmsted Point, Little Meadow, and Crane Flat. Significant codes are as follows: p-value < 0.001 = *, 0.01= **,0.0125 =*.**

Population	Model	Df	Sum Sq	Mean Sq	F-value	p-value
Yosemite Creek	LG8b Genotype	2	0.182	0.0912	0.132	0.878
	Residulals	14	9.7	0.6929		
Olmsted Point	LG8b Genotype	2	0.78	0.3889	0.78	0.458
	Residulals	250	124.25	0.497		
Little Meadow	LG8b Genotype	2	0.201	0.1004	0.93	0.403
	Residulals	39	4.204	0.1078		
Crane Flat	LG8b Genotype	2	3.19	1.594	3.23	0.0443
	Residulals	88	43.43	0.4935		

Historically there has been much debate in the literature about the genetic architecture of species differences. Should species differences be controlled by many loci of small effect (Fisher 1930) or by a few large effect genetic changes (Gould 1980, Gottlieb 1984)? In recent years special attention has been paid to the genetic architecture of species or population differences in the presence of gene flow, particularly during speciation (reviewed in Nosil and Feder 2012). A recent theoretical model incorporating drift, selection, and migration finds that when populations diverge with gene flow there should be few genetic loci of large effect involved (Yeaman and Whitlock 2011). Our two populations, SHL (*M. laciniatus*) and SHG (*M. guttatus*), occur within a meter of each

other and thus are very likely exchanging genes despite differences in mating system. Our data supports the idea that when there is gene flow between species, species differences are maintained by a few large effect loci or a few regions of many linked small effect loci (Yeaman and Whitlock 2011). The rest of the genome is allowed to interbreed and recombine freely.

An interesting pattern that emerged from this common garden experiment is that our large effect pleiotropic QTL, LG8b, is positive for flowering and floral traits, but negative for leaf shape (Table 11). In other words at LG8b the *M. guttatus* allele increases flowering time, node of first flower, and flower size as we would expect from the parental distributions of these traits, but the *M. guttatus* allele also increases leaf lobing. This is unexpected since *M. guttatus* possesses un-lobed, or entire, leaves. It does not seem to be a genotyping error because we found one leaf shape QTL, LG2 that overlaps with our bulk segregant leaf shape mapping, with the same analysis that is in the correct direction. Also we find the same pattern in our phenotypic correlation analysis (Table 10). Leaf lobing is positively correlated with floral size and flowering traits, meaning that in our F2 population plants with highly lobed leaves had larger flowers and flowered later than plants with entire leaves. From the parental distributions of traits we would expect a negative correlation between leaf lobing and flowering/floral traits (Figures 14 & 15).

What is creating this positive genotypic and phenotypic correlation between later flowering, large flowers, and lobed leaves is difficult to say. It seems highly unlikely that it is a segregating polymorphism in the *M. guttatus* population. *M. guttatus* does often have small feathery projections at the base of its leaves, but to this author's knowledge truly lobed leaves have only been observed in the M2L population of *M. guttatus* and those are not nearly as lobed as *M. laciniatus's* leaves (see Chapter 3). We also found that lobed leaves were dominant to entire leaves in our previous bulk segregant analysis so a lobed leaf shape allele in our SHG parent would have to be recessive. It should not be due to recent hybridization between our *M. laciniatus* and *M. guttatus* parents because then the genotype should resemble SHL more than SHG. Perhaps this negative QTL is an epistatic interaction between an *M. guttatus* allele at LG8b and an *M. laciniatus* allele elsewhere in the genome. Further studies are needed to investigate the link between negative leaf shape and positive flowering and floral size association at LG8b.

4.5.2 Local Adaptation and Phenotypic Selection

Since the early days of reciprocal transplant experiments with Clausen, Keck, and Hiesey local adaptation between populations and species has been repeatedly demonstrated in plants (reviewed in Hereford 2009). However the traits that are responsible for this differential adaptation and the genes that underlie them are still poorly understood. Using genetic manipulation to separate a trait from its genetic

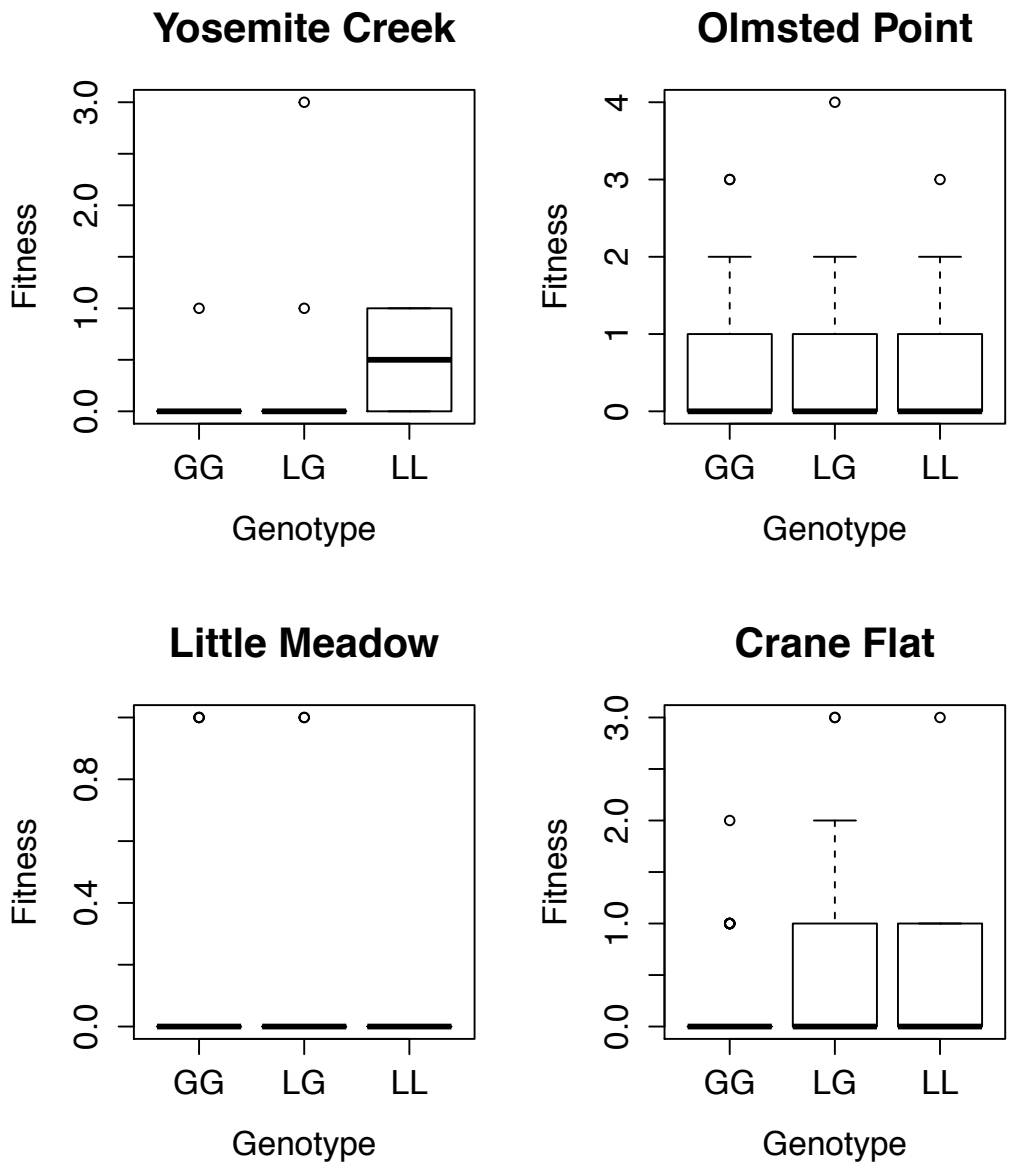

Figure 19. Plots of genotype at our pleiotropic QTL on LG8 vs. fitness at each of the four reciprocal transplant sites: granite outcrop sites are Yosemite Creek and Olmstead Point while Little Meadow and Crane Flat are meadow habitat.

background makes it possible to measure its individual adaptive significance. In order to test the adaptive significance of early flowering time, small flower size associated with a self-fertilizing mating system, and lobed leaf shape in *M. laciniatus* we performed a reciprocal transplant in the Sierra Nevada Mountains of California with an experimental *M. laciniatus x M. guttatus* F4 population. We found several interesting patterns.

First of all we find that *M. laciniatus* has significantly higher fitness than *M. guttatus* in the granite outcrop habitat. *M. laciniatus* parents were significantly more likely to survive to flowering and produce more fruits in the granite outcrop than *M. guttatus* parents (Table 16, Figure 18). In the meadow habitat we saw little difference in survival to flowering between *M. laciniatus* and *M. guttatus*, but *M. guttatus* produced more fruits in its native meadow habitat although this difference was not significant (Figure 18). This lack of significance in fecundity in the meadow habitat could be due to low power in our analysis since overall both species performed worse in the meadows than in the granite outcrops reducing our sample sizes in this habitat. An earlier reciprocal transplant study of *M. laciniatus* and *M. guttatus* found that *M. laciniatus* had a higher rate of survival to flowering in granite than *M. guttatus*, but that the two seemed to do equally well in the meadow site (Peterson et al 2011). Our results agree with theirs in this respect, but their study did not measure fecundity. Many reciprocal transplant

studies have found that one species or population does best in both habitats. This is Our fruit number results suggest that there may be a trade-off in lifetime fitness between these two species in their native environments that was not observable with survival data alone.

Because of the rapid and early onset of seasonal drought in granite outcrops in the Sierra Nevada Mountains (Peterson et al 2010) early flowering time should be advantageous in this habitat. In our selection analysis we found evidence of directional selection on earlier flowering time and larger flower width in both granite outcrop and meadow habitats. However selection for earlier flowering time was only significant in the granite habitat and there was a significant interaction between flowering time and habitat type in our model indicating differential selection habitats (Tables 17 & 15). These results confirm our hypothesis that early flowering time is adaptive in *M. laciniatus's* granite outcrop habitat. Many previous studies have found selection for earlier flowering time in annual plants that occupy seasonally dry environments (Kiang and Hamrick 1978, Fox 1990, Eckhart et al 2004, Hall and Willis 2006). In perennial plants growing in moist environments we expect selection for later flowering or stabilizing selection on midseason flowering since later flowering plants are larger and tend to have higher fecundity (Mitchell-Olds 1996, Hall and Willis 2006, Anderson et al 2012). However the *M. guttatus* parent in our cross is an annual and selection for earlier

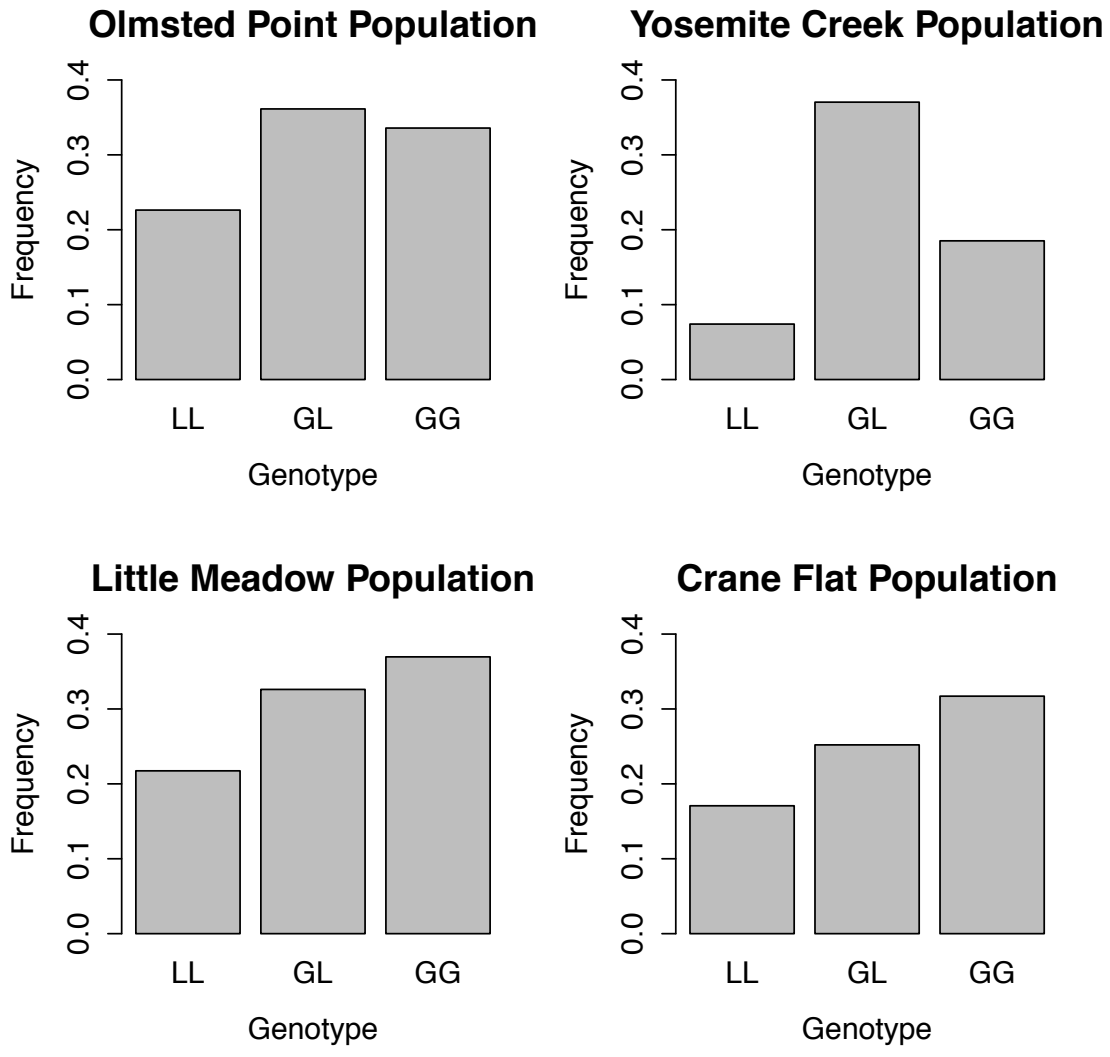

Figure 20. Bar graphs of genotype frequency at the LG8b pleiotropic QTL in our four reciprocal transplant sites: Olmsted Point, Yosemite Creek, Little Meadow, and Crane Flat.

flowering has been previously found in annual populations of this species (Hall and Willis 2006). In addition, while there was negative selection on flowering time in both

habitats F4's still flowered later on average in the meadow sites than in granite outcrops (Table 12).

Positive directional selection on flower size was expected in the meadow habitats because *M. guttatus* has large flowers and is highly outcrossing. Therefore it seems likely that larger flowers that can better attract pollinators would be advantageous in its native habitat. However we also saw selection for larger flowers in the *M. laciniatus* habitat which was unexpected since the native plants are highly self-fertilizing and small flowered. We in fact observed fewer potential pollinators at our granite sites than in the meadow transplant populations suggesting that self-fertilization is a more reliable strategy in granite outcrops. However, perhaps increased flower size facilitated an increased rate of outcrossing via pollinator visitation and was advantageous in both habitats since our experimental plants are derived from inbred lines. If we had analyzed seed number instead of fruit number then selection for larger flowers might have been expected in both habitats because larger flowers tend to produce larger fruits with more seeds. However this should not affect our analysis since we looked at fruit number which does not obviously correlate with flower size.

We found no find significant directional selection on leaf lobing in either of the granite outcrop sites, but we did find positive directional selection on leaf shape in the Crane Flat meadow. Positive directional selection on leaf lobing is unexpected in Crane

Figure 21. Digitally scanned first true leaves from experimental plants in the Yosemite Creek transplant site.

Flat since *M. guttatus* has entire round leaves, but one potential reason for this result is that plants in Crane Flat were by far the largest, in both height and leaf area, of any site (Table 13). Larger plants tend to develop more lobed leaves. Therefore, there may have simply been more plants with truly lobed leaves in Crane Flat than in any other site. Our results from this experiment cannot currently reject or confirm our hypothesis about the adaptive significant of leaf shape.

In our reciprocal transplant we also found phenotypic plasticity in flowering time, plant height, flower width, and leaf area between granite and meadow habitats. Plants in all genotypic classes (F4, *M. laciniatus*, *M. guttatus*) displayed earlier flowering, were shorter and had smaller leaves in the granite habitat (Table 12). This plasticity is in the expected direction of adaptation since earlier flowering and smaller plants with smaller leaf areas should be advantageous in rapidly drying habitats like granite outcrops (Parkhurst et al 1968, Fox 1990, Macnair and Gardner 1998, Schuepp 1999, Nobel 2005). From our above selection analysis we can conclude that both *M. laciniatus* and *M. guttatus* exhibit adaptive phenotypic plasticity in flowering time because both parental species flowered earlier in the granite habitat where selection for early flowering time was strongest. The direction of plasticity in flower width was unexpected. In the F4's and *M. laciniatus* parental line, flowers were wider in the granite outcrop habitat than in the meadows. *M. laciniatus* is highly self-fertilizing and has small

flowers which may be adaptive because of lower water loss in floral tissue and the lack of need to attract pollinators. If small flowers are adaptive in granite outcrops then we might expect phenotypic plasticity to be in that direction. Leaf shape did not exhibit much plasticity between field sites in the F4's or *M. guttatus*. There was variation in leaf shape among *M. laciniatus* between populations, but it was not in a consistent direction between habitats. In fact, we saw very little variation in leaf shape either within or between populations in our field experiment. Very few experimental plants, even *M. laciniatus*, possessed truly lobed leaves and all had very small leaves (Figure 21). This was also true in the native *M. laciniatus* populations at both Olmsted Point and Yosemite Creek.

The year we performed this field experiment, 2013, was one of the driest years on record in California evidenced by the Yosemite Rim Fire that took place at the end of the summer and was one of the largest wildfires in California's history. This has no doubt played a role in the results of our selection analyses. In past field seasons native *M. laciniatus* populations displayed a varying level of leaf lobing, but always significantly more than was observed in 2013. The severe drought seems to have altered leaf shape so that *M. laciniatus* leaves were unusually small (Figure 21). It has been observed by the author that, both in the field and greenhouse, the smaller the leaf the less lobed it is likely to be. This extremely dry weather may have also contributed to the fact that

overall plant survival and fecundity was lower in the meadow sites than in the granite outcrops. The meadow habitat sites we chose are 2000ft lower in elevation than the granite outcrop sites and effects of the drought were more pronounced at lower elevations. Our meadow sites dried out more quickly than expected for *M. guttatus* habitat and even the native *M. guttatus* populations were visibly stressed. The lack of variation in leaf lobing in native and experimental plants in 2013 made it an unfortunate season to test the adaptive significance of leaf shape in granite outcrops.

4.5.3 Pleiotropic greenhouse QTL has mixed effects on fitness

In our reciprocal transplant experiment we found crossing reaction norms between *M. guttatus* and *M. laciniatus* for fruit production in their native habitats. In order to test whether this fitness trade-off was due to antagonistic pleiotropy at a single locus we looked for an association between fitness in our F4 population and genotype at the large effect pleiotropic QTL, LG8b, that we found in our greenhouse experiment. If a fitness trade-off between these species were due to antagonistic pleiotropy at this locus we would expect the *M. laciniatus* homozygotes to have higher fitness in granite outcrops, but lower in meadow habitat. And we would expect the opposite pattern for *M. guttatus* homozygotes. However, after correction for multiple testing we did not find a significant relationship between LG8b genotype and fruit number at any of our reciprocal transplant sites (Table 18). From this analysis it seems unlikely that our

greenhouse QTL at LG8b is involved in fecundity in the field. This is surprising since LG8b genotype had a large effect on flowering time in the greenhouse (Table 11) and in our phenotypic selection analysis we found that earlier flowering time significantly increased fecundity in the field (Table 16).

Several things may account for our results not matching our expectations. It is first of all possible that our statistical analysis lacked power we do to our small genetic sample sizes. Second of all LG8b was mapped in an F2 population created from an entirely different cross (SHL x SHG) than was used to construct our field F4 population (WLF x YVO). Different populations contain varying subsets of a species' genetic variation. Due to increased homozygosity and reduced gene flow between populations self-fertilizing species like *M. laciniatus* should harbor more genetic diversity between populations than within them (Wright and Charlesworth 2001). Therefore it is possible that different QTL's underlie flowering time among populations of *M. laciniatus* and that consequently LG8b does not contribute to genetic variation in flowering time in our F4 population. A third possibility is that our QTL at LG8b is not stable across all environments. There are many instances in the literature of mapping different QTLs for the same trait across different environments (Paterson et al 1991). This phenomenon is attributed to genotype-by-environment interactions which are common in plants (reviewed in Des Marais et al 2013).

In order to see whether genotype at the LG8b locus affected survival to flowering we looked for skew in the genotype frequencies at each experimental site. If there was no selection on LG8b before flowering then we should see a 1:2:1 ratio of genotypes in each population. However, if for example *M. laciniatus* homozygotes were more likely to flower in granite outcrops and *M. guttatus* homozygotes were more likely to flower in meadows, we would see an excess of the local homozygote. We found significant differences between our observed and expected genotype frequencies at two of our reciprocal transplant sites, Olmsted Point (granite) and Crane Flat (meadow). When we plot genotype frequencies at each site there appears to be an excess of the local *M. guttatus* homozygote in both meadow habitats: Little Meadow and Crane Flat. However in the granite outcrop sites there is no pattern of local homozygote excess and we in fact find an excess of foreign *M. guttatus* homozygotes at Olmsted Point (Figure 20). These results indicate that *M. guttatus* homozygotes at LG8b experienced a selective advantage before flowering in granite (Olmsted Point) and meadow (Crane Flat) populations. This is not the pattern of selection we expect to see at a locus that contributes to differential habitat adaptation between *M. laciniatus* and *M. guttatus* through antagonistic pleiotropy. From these results we conclude that while LG8b does contribute to phenotypic divergence in many traits between *M. laciniatus* and *M. guttatus* populations in the greenhouse, it does not contribute to differential habitat adaptation in the field via

antagonistic pleiotropy. One caveat to this conclusion is that we did not empirically measure the genotype frequency at LG8b in our F4 population before selection and we therefore cannot be positive that it was originally at equilibrium frequency (1:2:1). There may have been segregation distortion at this locus that created an excess of *M. guttatus* homozygotes in our original F4 population.

4.5.4 Conclusions

Understanding which traits contribute to differential habitat adaptation, and the nature of their genetic architecture, has been a long-standing goal in evolutionary biology. In this study we have found that a few large effect pleiotropic QTL's underlie divergence in life history and morphological traits between sympatric *M. laciniatus* and *M. guttatus* populations, that *M. laciniatus* is better adapted to its granite outcrop environment than its close relative *M. guttatus,* and that earlier flowering time contributes substantially to this adaptation. The highly pleiotropic nature of our largest effect QTL, LG8b, is significant since the degree of pleiotropy impacts the efficacy of selection on individual traits (Lande 1979). Our results support recent theory (Yeaman and Whitlock 2011) that when there is gene flow between populations, few loci of large effect should control adaptive differences between species.

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Biography

I was born on November 23rd 1984 in St. Louis, MO. I did my undergraduate work at the University of Chicago where I double majored in Biological Sciences and English Language and Literature. I received my bachelors degrees in June of 2007. I have published one scholarly article: Sexton, J. P., K. G. Ferris, and S. E. Schoenig. 2013. The fern-leaved monkeyflower (Phrymaceae), a new species from the northern Sierra Nevada of California. *Madroño* 60:236–242. I have received an NSF Doctoral Dissertation Improvement Grant, a Sigma Xi Grant-in-Aid of Research, two Duke Department of Biology Grants-in-Aid of Research, and a California Native Plant Society Education Grant. I am a member of the American Naturalists Society and the Society for the Study of Evolution.